Synthesis and biological evaluation of carbohydrate-mimetics as ligands for Siglecs

Inauguraldissertation

zur Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

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aus Rickenbach, Deutschland

Basel, Dezember 2011

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Basel, den 22. Juni 2010

Prof. Dr. Eberhard Parlow
Dekan

Ever tried?
Ever failed?
No matter.
Try again.
Fail again.
Fail better.

(Samuel Beckett)

Acknowledgement.

I would like to thank Prof. Beat Ernst for giving me the opportunity to perform my PhD thesis under his supervision. I am grateful for his continuous support and advice, the scientific discussions and for the nice environment at the IMP.

I also want to thank Prof. Bernd Giese for accepting to be the co-referee of my PhD defense.

Many thanks to Oliver Schwardt for performing the HR-MS measurements, his help with the LC-MS and his scientific advises. Furthermore, I want to thank Bea Wagner for her help with all chemistry-related tasks, for introducing me to the IMP and the organization of lab-related tasks. I am indebted to Delia Moser, who performed her master thesis under my supervision for her synthetic contributions to the project and for being a friend. Furthermore, I would like to thank Dr. Sachin Shelke for his help at the beginning of my master thesis and I am grateful to Dr. Xiaohua Jiang for her collaboration in the MAG project. Many thanks to Dr. Brian Cutting for his support and patience while introducing me to the NMR. I am grateful to Dr. Daniel Strasser for introducing me to the Biacore and for setting up the MAG-assay and to Celine Weckerle for numerous discussions and help. I want to thank Katrin Lemme for the ITC-measurements, her friendship and her engagement in IMP-internal organization. I am grateful to Matthias Witter for the measurements of the various pharmacokinetic parameters of my antagonists and furthermore for his continuous chocolate support. Thanks to Roland Preston and Dr. Daniela Stockmaier for protein quality test by SDS pages and Western-blots.

Especially, I want to thank Jonas Egger for his support and patience during three and a half years, the countless (non-)scientific discussions and his companionship in daily caffeine take-up. Many thanks go to the modeling team, Prof. Angelo Vedani, Dr. Morena Spreafico and Gianluca Rossato and to their support and their efforts with the MAG homology model and all subsequent modeling work.

I want to thank Prof. Soerge Kelm, Heiko Gaethke and especially Hendrik Koliwer-Brandl for the supply of MAG and CD22, and for the measurement of the hapten inhibition assay. Tanks also to Prof. Paul Crocker and Johanna Tokarzewska-Zadora for supplying my Sialoadhesin and Siglec-7.

I want to thank all IMP members for the nice working atmosphere and many thanks to my friends and colleagues for nice time outside the lab and for numerous impressive moments.

Finally, I want to thank my family for their support and patience for the last (almost) 30 years.

Abstract.

Sialic acid binding immunoglobulin-like lectins (Siglecs) play an important role in the mediation of cell-cell interactions as well as in the regulation of signaling pathways. They are mainly expressed in the haematopoietic and immune system, with exception of Siglec-4, also called myelin-associated glycoprotein (MAG). It was identified as one of neurite outgrowth inhibitors, playing a crucial role in paraplegia, which is caused by injuries of the central nervous system (CNS) and especially young people suffer from these severe consequences as, for example, the loss of motor functions. The lack of repair of the injured nerve strands originates from the inhibitory environment for axon regeneration in the CNS. Specific inhibitory proteins, such as MAG block the regrowth of nerve roots. We identified potent small molecule MAG antagonists modifies in the 2- and 5-position. Furthermore, we investigated new neuraminic acid derivatives modified in the 4-position, and the influence of various structural modifications on their kinetic and thermodynamic binding properties. In a next step we presented high affinity ligands, which were identified in second-site screenings and optimized them according to medicinal chemistry aspects. All ligands were elucidated with respect to their binding affinity as well as their kinetic and thermodynamic profile.

Siglec-2, also known as CD22, is involved in the regulation and survival of B-cells and has been successfully targeted in cell depletion therapies with antibody-based approaches. Sialic acid derivatives, already known to bind with high affinity to myelin-associated glycoprotein (MAG, Siglec-4), were screened for their binding affinity for CD22 by surface plasmon resonance. The best compound identified was further modified with various hydrophobic substituents at the 2-, 5-, and 9-positions of the sialic acid scaffold, leading to nanomolar derivatives. Furthermore, initial tests regarding drug-like properties of these antagonists demonstrate the required high plasma protein binding yet a lack of oral availability, although its distribution coefficient (log D) is in the required range.

Finally, we investigated a library of sialic acid mimetics with respect to binding towards another member of the Siglec family, namely Sialadhesin and discuss the influence of various structural moieties with regard to the arising selectivity towards these three proteins.

Abbreviations.

$[\alpha]_D^{20}$	optical rotary power
Ac	acetate
AIBN	α,α'-azodiisobutyronitrile
aq	aqueous
BBB	blood-brain barrier
BCR	B-cell receptor
Bn	benzyl
CHO	chinese hamster ovary
ClAc	chloroacetyl
CMC	critical micelle concentration
CNS	central nervous system
DCE	1,2-dichloroethane
DCM	dichloromethane
DIPEA	*N,N*-diisopropylethylamine
DMAc	dimethylacetamide
DMAP	4-dimethylamino-pyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
EDC	3-(*N,N*-dimethylamino)propyl-*N*-ethylcarbodiimide
FAc	fluoroacetyl
Gal	galactose
Gal*N*Ac	*N*-acetylgalactosamine
GIT	gastrointestinal tract
Glc	glucose
GRB2	growth factor receptor-bound protein 2
HBS-E	HEPES/NaCl/EDTA buffer
HBS-EP	HEPES/NaCl/EDTA/P20 buffer
IgG	immunoglobuline G
*i*PrOH	2-propanol
ITAM	immunoreceptor tyrosine-based activation motif
ITC	isothermal titration calorimetry
ITIM	immunoreceptor tyrosine-based inhibitory motif

K_A	association constant
K_D	dissociation constant
(HR)MS	mass spectrometry
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
NgR	Nogo receptor
NHS	*N*-hydroxysuccinimide
NIS	*N*-iodosuccinimide
NMR	nuclear magnetic resonance
Nosyl	2-nitrobenzylsulfonoyl
PAMPA	parallel artificial membrane penetration assay
PDB	protein data bank
PDC	pyridinium dichromate
PPB	plasma protein binding
PPTS	pyridinium *p*-toluenesulfonate
R	gas constant
rIC_{50}	relative half maximal inhibitory concentration
RP	reversed phase
SAR	structure-affinity relationship
Sat	saturated
Siglec	sialic acid immunoglobuline-like lectin
SPR	surface plasmon resonance
STD	saturation transfer difference
T1rho	longitudinal relaxation
^{t}BuOH	*tert*-butanol
TBS	*tert*-butyltrimethylsilyl
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TfOH	trifluoroacetic acid
THF	tetrahydrofurane
TMS	trimethylsilyl
trNOE	transfer Nuclear Overhauser Enhancement
TsCl	*p*-tolylsulfonyl chloride
Z (Cbz)	carboxy benzyl

Table of Content.

1. Introduction

1.1 Carbohydrate-binding proteins.

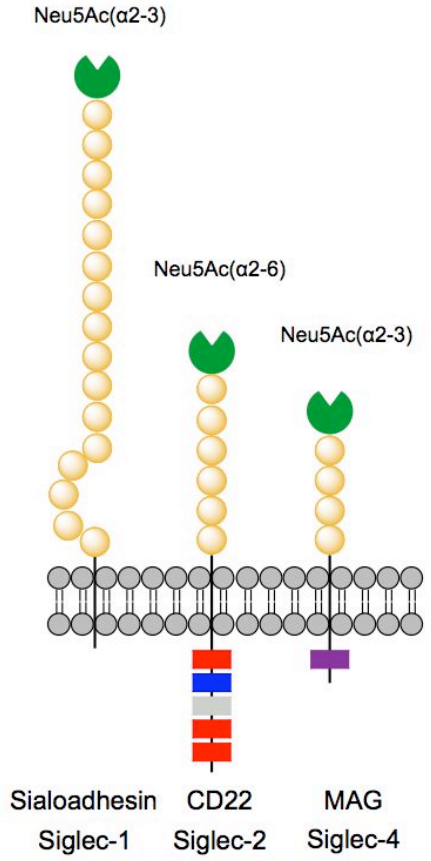
Carbohydrates are involved in numerous physiological processes. Complex carbohydrates, so called glycans, are located on cell surfaces and serve as ligands for protein-carbohydrate interactions, *e.g.* they act as regulatory elements in the immune and nervous system or regulate metabolic processes.[1] For many of these functions, sialic acid, which is found as terminal carbohydrate moiety of glycan structures on glycoproteins or glycolipids, plays an important role.[2] Until recently, the modulation of carbohydrate-protein interactions has rarely been exploited in medicinal chemistry. However, in the last decade, the interest steadily increased and nowadays, intensive research is focusing on the pharmaceutical potential of carbohydrate-protein interactions.

The focus of our investigations are members of the Siglec family. Three members of this family of sialic acid binding lectins will be discussed with respect to their physiological functions and the current status of antagonist research.

1.2 Siglecs.

The immunoglobulin (Ig) superfamily comprises a distinct subgroup of proteins, which share structural and functional similarities and bind to sialic acid by definition. They were classified as **s**ialic **a**cid binding immuno**g**lobulin-like **l**ectins (Siglecs).[3-5] Siglecs are type 1 membrane proteins which have an *N*-terminal V-set immunoglobulin domain, responsible for sialic acid binding and a variable number of C2-set immunoglobulin domains.[6] According to their structural similarity, they are divided into two subsets: Sialoadhesin (Siglec-1), CD22 (Siglec-2), Myelin-associated glycoprotein (MAG, Siglec-4) and Siglec-15 are highly conserved proteins in mammalian species and show some structural similarities (25-30%) in their sequences, whereas the CD33-related Siglecs (Siglec 3, 5-14) show similarities of about 50-90%.[4,5] Up to now, detailed structural information is available for Sialoadhesin[7,8] and Siglec-7.[9,10] The binding site for sialic acid was found to be highly conserved, however the different members display specificities for a distinct sialic acid linkage to the residual glycan structure. Furthermore, they are expressed in a highly constrained manner, suggesting that the proteins fulfill distinct functions. With exception of MAG and Siglec-6, Siglecs are mainly expressed in the haematopoietic and immune system. In general, they are involved in the mediation of cell-cell interactions as well as in the regulation of signaling pathways. Some of them have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytosolic part

(*e.g.* CD22), enabling them to suppress activation signals by recruitment of tyrosine and inositol phosphatases.[4]



Siglec	Cell-type expression	Disease relevance
Siglec-1	Tissue Macrophages	HIV infection
Siglec-2	B cells	Lymphoma leukemia, SLE rheumatic arthritis
Siglec-4	Oligodentrocytes Schwann cells	Paraplegia

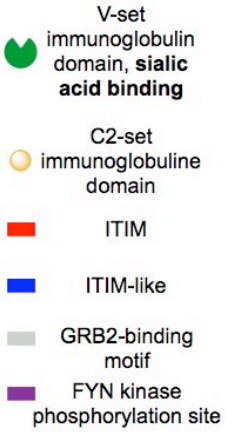


Figure 1. Schematic representation of the structure of Sialoadhesin, CD22 and MAG; GRB2: growth-factor-receptor bound protein 2; ITIM: immunoreceptor tyrosine-based inhibitory motif; adapted from Crocker *et al*, 2007.[4]

Here, only the first subgroup will be discussed in more detail. For CD33-related Siglecs von Gunten *et al.* recently published a review.[11]

Sialoadhesin (Siglec-1) is the largest member within the Siglec-family, having 17 extracellular domains, and is expressed on macrophages. As it lacks the ITIM motif, it is predominantly involved in cell-cell interactions.[12,13] Furthermore, the interspace between the binding site of Sialoadhesin and cell surfaces excludes masking by *cis* ligands, a phenomena observed with all other Siglecs.

Whereas the expression of Sialoadhesin is normally restricted to subpopulations of macrophages in lymphoid tissues,[13] Sialoadhesin is highly up-regulated on inflammatory

macrophages, which act as effectors in inflammation associated with autoimmune diseases.[13] Moreover, tumor cells secrete macrophage-activating factors, stimulating inflammation and facilitating tumor spread.[14] First, modulation of macrophages expressing Sialoadhesin might be an interesting approach for the treatment of inflammation related to tumor spread. But more importantly, Sialoadhesin-mediated interactions were shown to be important for effective killing of tumor cells by CTLs.[15] In addition, increasing interest evolved in Sialoadhesin, as it was observed to play an important role in HIV and bacterial infection. Sialoadhesin binds to HIV-1 directly and is responsible for trans-infection.[16] Furthermore, Sialoadhesin is overexpressed on CD14+ monocytes correlating with HIV-1 viral load.[17] Remarkably, it was found that interactions occur in a sialic acid dependent as well as independent manner. In the first case, interactions are mediated by sialomucins (*e.g.* CD43,[18] mucin-1[19]), which contain high densities of sialylated *O*-linked glycans with terminal (α2-3) linked sialic acids.[20] CD43, considered as putative counter-receptor of T-cells for Sialoadhesin, enables initial physical contacts between macrophages and T-cells.[18] In contrast, sialic acid independent pathways may involve *e.g.* binding to mannose receptor.[4]

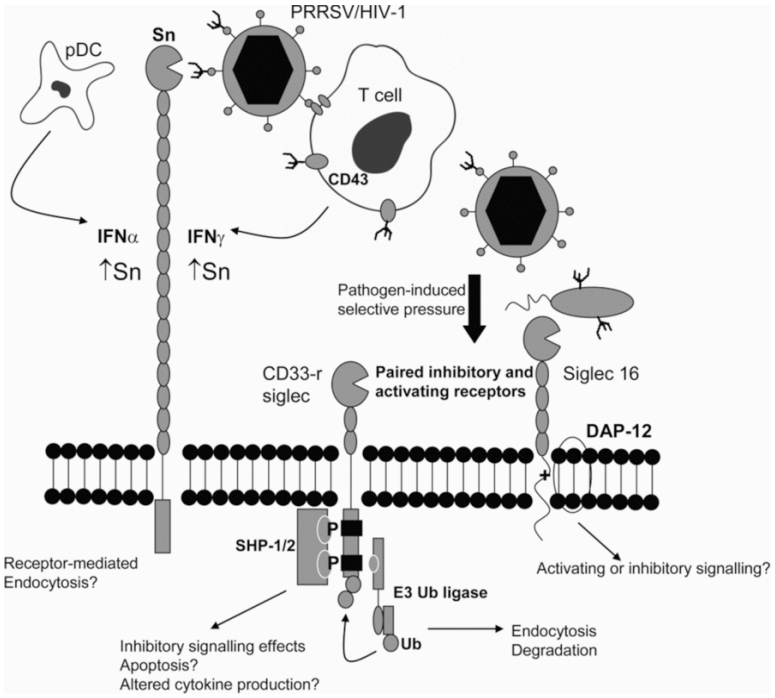


Figure 2. Sialoadhesin is involved in cell-cell interaction and recognition, Crocker *et al.*, **2008**.[6]

CD22 (Siglec-2) is expressed on the surface of B-cells and B-cell lymphomas. It is involved in the regulation of B-cell activity,[21-23] homeostasis[24] and survival.[25] CD22 was identified as inhibitory co-receptor for B-cell receptors (BCR), being therefore responsible for a threshold for B-cell activation and thereby preventing autoimmune responses.[22,26] Upon antigen-

induced stimulation, BCR-ligated CD22 is phosphorylated at its ITIM motifs, initiating a cascade, which finally leads to a dampening of the BCR-induced signal. The inhibitory activity of CD22 depends on binding to BCR and on the interaction with glycans on the cell surface (= *cis* ligands). This has been demonstrated with CD22-deficient mice, showing hyperinflammation,[27-29] whereas mice lacking *cis* ligands of CD22 showed the adverse effect, namely hypoinflammation.[30] In the latter case, it was also found that the allocation of BCR was changed, being more clustered with CD22 than in the wild-type[4,31] and consequently being faster internalized by endocytosis. CD22 has several ITIM motifs in its cytosolic part, but also an activating GBR2-motif. The ITIM motifs are crucial for initiating the inhibitory cascade and are also required for clathrin-dependent endocytosis.[26] Consistently, CD22 is primarily located in clathrin-coated pits, and undergoes constitutive endocytosis.[31,32]

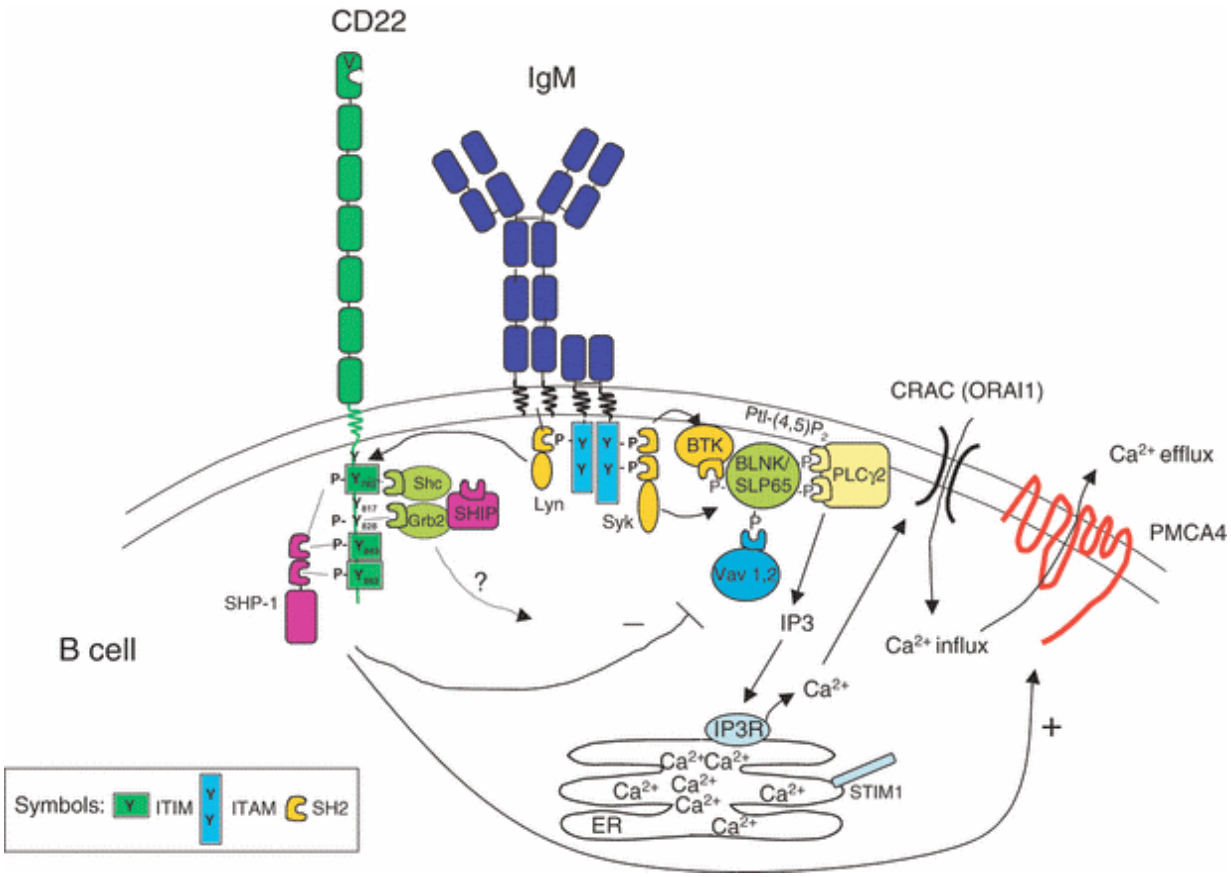


Figure 3. Regulation of B-cell activity by CD22, from Nitschke *et al*, **2009**.[26]

CD22 has a specificity for Neu5Acα2-6Gal, binding with an affinity of 100-200 µM.[20,33] Since the glycan concentration on B-cell surfaces is estimated to be around 25-30 mM, CD22 is constitutively masked,[34] *i.e.* its binding site is blocked by sialylated ligands located on the cell surface. This "masking" by *cis* interactions exhibits a threshold for CD22, which can be

competed by *trans* ligands with sufficient high affinity and avidity. Collins *et al.* reported that upon binding to cells bearing CD22 ligands, CD22 clusters at the site of contact and thereby increases its local concentration.[34] Interestingly, Razi *et al.* reported that CD22 gets unmasked upon activation of B-cells, *e.g.* using protein kinase C activation.[35] Furthermore, certain sub-populations of B-cells bear constitutively un-capped CD22 which interacts with ligands expressed on the sinusoidal endothelium leading to subsequent homing of B-cells to the bone marrow.[36,37]

CD22 has become a target for cell depletion therapies as it was proven to be a marker for B cell malignancies[38-40]. Moreover, it plays an important role in lymphoma, AML (leukemia) and non-Hodgkins lymphoma (NHL)[41]. Clinical trials with anti-CD22 antibodies are ongoing, two of them being immunotoxins[42,43], and one being a native antibody, called epratuzumab[44]. The immunotoxin approach is based on antibody-triggered endocytosis, leading to selective internalization of antibody-coupled toxins. BL22 is an anti-CD22 conjugated exotoxin, being in clinical phase I/II[42] and CMC-544 is a humanized IgG4 anti-CD22 antibody conjugated to the chemotoxin calicheamicin[43], and is in clinical trials phase II/III. Epratuzumab alone shows only modest reduction of B-cells, however in combination with the anti-CD20 antibody rituximab promising results have been reported[44]. Furthermore, it was observed that blocking the binding sites of CD22 effects the half-life time and turnover of B-cells[45]. Recently, the concept of targeting CD22 for triggering endocytosis is an arising field using synthetic sialosides. Finally, CD22 is regarded as a promising target for modulation antigen-induced responses.

Myelin associated glycoprotein (MAG, Siglec-4) is expressed on oligodentrocytes in the central nervous system (CNS) and plays a crucial role in myelin stabilization as well as in the inhibition of nerve strand outgrowth in adults after injury[46-49], causing paraplegia. The lack of regeneration originates from the inhibitory environment in the CNS, *i.e.* specific inhibitor proteins on residual myelin and on astrocytes, which are recruited to the site of injury. A therapy for full regeneration of injured nerve strands is not yet available. However, in the last decade, several of these inhibitor proteins have been identified, one of them being the myelin-associated glycoprotein (MAG). Other inhibitory proteins are Nogo-66[50] and oligodentrocyte myelin-associated glycoprotein (OMgp)[51]. They all bind to the same Nogo-66 receptor (NgR), followed by complex formation with the transmembrane neurotrophin receptor p75 ($p75^{NTR}$)[52]. Although the exact mechanism of the signaling pathway remains to be determined, it has been demonstrated that Nogo, MAG and OMgp activate the small

GTPase Rho-A in a p75-dependent manner, inducing subsequent growth cone collapse[53,54]. Interaction of MAG with NgR has been reported to be sialic acid independent, however, Venkatesh *et al.* have recently shown that the Nogo receptor homolog NgR2 is a sialic acid dependent receptor for MAG[55]. As a second pathway, it was proposed that MAG interacts with gangliosides, *e.g.* GD1a, GT1b and GQ1bα, which are predominantly found on neuron cell surfaces in the CNS[56-58]. These gangliosides were proposed to be function-mediating binding partners of MAG. By clustering of GT1b with a multivalent IgM antibody, the inhibitory effects of MAG via signaling to the intracellular mediator Rho-A was mimicked[59,60].

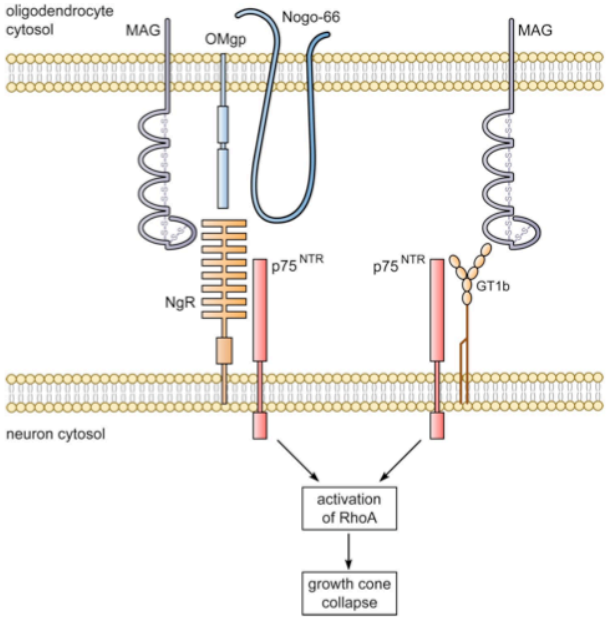
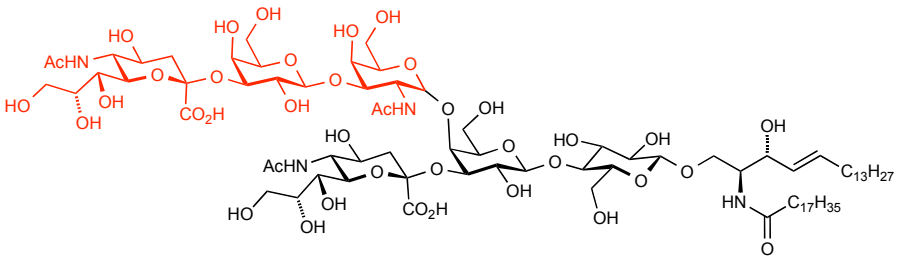


Figure 4. Signaling cascades of MAG, involving interaction with NgR or gangliosides, adapted from Filbin*et al.*[61] by A. Vögtli.

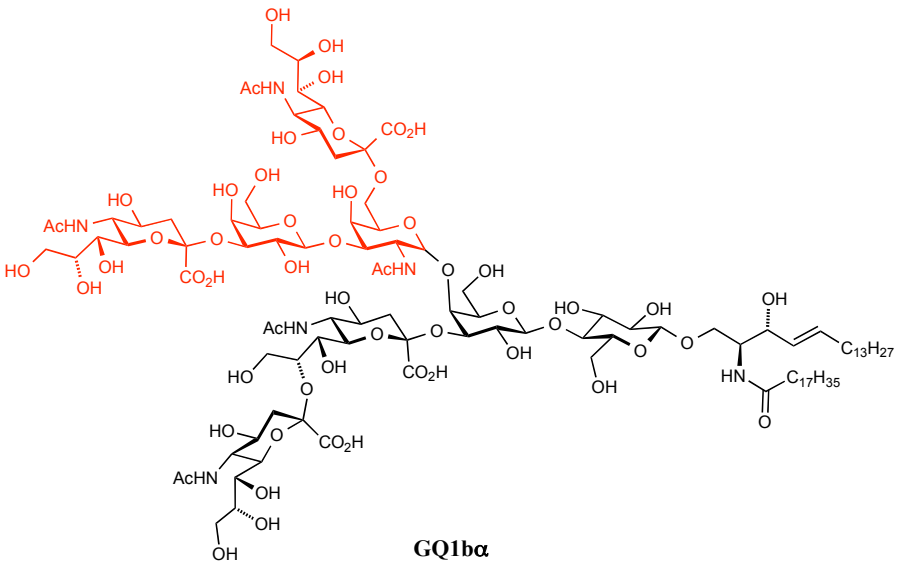
Although the relative role of Nogo receptors and gangliosides as MAG ligands has yet to be resolved, in some systems, neurite outgrowth can be initiated by sialidase treatment, suggesting that the sialic acid-mediated interactions of MAG, *i.e.* the interaction with gangliosides, predominantly contributes to the inhibitory process. Furthermore, the ability to reverse MAG-mediated inhibition in an affinity-dependent manner was shown for a partial structure of GQ1bα[62,63]. Therefore, blocking MAG with potent glycomimetic antagonists offers a valuable therapeutic approach for the enhancement of axon regeneration.

1.3 Gangliosides.

Gangliosides, glycosphingolipids with terminal sialic acids, are the natural ligands of Siglecs. They are expressed on all vertebral cells, most abundantly on the surface of nerve cells and in the hematopoietic lineage[1]. They are fixed to the plasma membrane by a ceramide lipid anchor, and based on the core of four uncharged sugar residues attached to ceramide they are divided into different sub-classes[64]. As they are located on the cell surface, they mediate cell-cell recognition and are involved in pathogen internalization. Moreover, they regulate signaling and response cascades in the immune system, influencing NK-cell cytotoxicity[65] and B-cell activity[3]. In the CNS, they are important receptors for Siglec-4 (MAG), stabilizing myelin-axon interactions and controlling the neurite outgrowth after injury[46]. As gangliosides play a critical role in immune regulation and signaling, they are an interesting target for therapeutic applications and, even more important, can serve as starting point for the development of therapeutic carbohydrate-antagonists and mimics thereof[66].



GD1a



GQ1bα

Figure 5. Structures of GD1a and GQ1bα, which are natural ligands for MAG,[67] but also bind to Sialoadhesin.[20]

Depending on the linkage of the terminal sialic acid, preferences for different Siglecs are established. Sialoadhesin (Siglec-1) and MAG (Siglec-4) prefer α2,3-linked sialic acid, whereas CD22 binds to α2,6-linked sialosides. Consistently, the glycans in the central nervous system bear predominantly α2,3- and on B-cells α2,6-linked sialic acids[20].

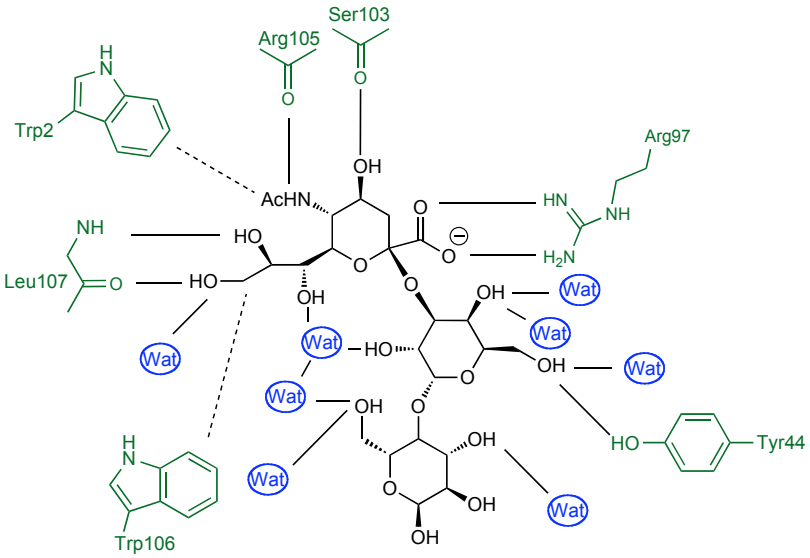
Table 1. Siglecs specificities and exemplary natural ligands.

Siglec	Preferred linkage	Physiological ligand
Siglec-1	α2,3	GQ1bα, GD1a
Siglec-2	α2,6	CD45*
Siglec-4	α2,3	GQ1bα, GD1a

*Glycoprotein, bearing α2-6-linked sialic acid

Nevertheless, the amino acids responsible for sialic acid binding were found to be conserved throughout the Siglec family. It was observed that the carboxylic acid is crucial for binding, as well as the glycerol side chain contributes strongly to the overall binding affinity[68,69]. Furthermore, different substituents at the 5-position of Neu5Ac are proposed to influence selectivity[8,70].

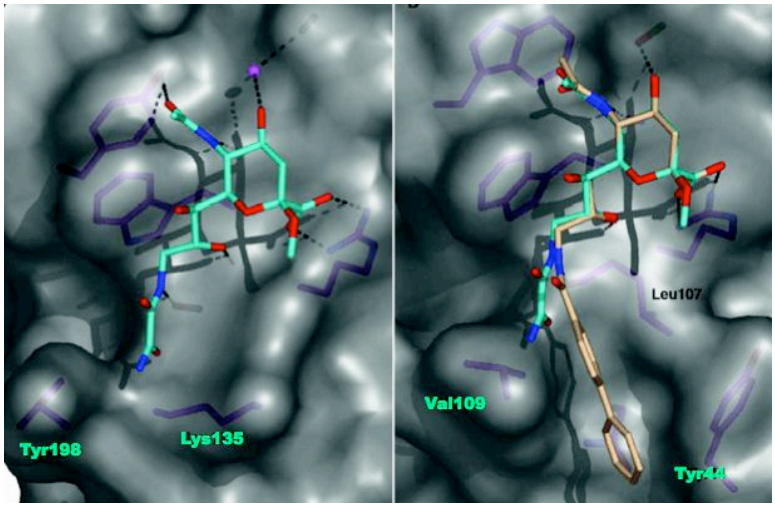
Sialoadhesin (Siglec-1) and Siglec-7 were co-crystallized with the trisaccharide Siaα2-3Galß1-4Glu (sialyllactose)[71] and a ganglioside analog DSLc4, 2-(trimethylsilyl)ethyl NeuAcα2,3Galβ1,3GlcNAcβ-[NeuAcα2,6]lactose,[72] respectively. Although these two proteins belong to different subgroups of the Siglec family, in both cases the most crucial interaction is a salt bridge formed between the carboxylic acid of Neu5Ac and Arg97 (Arg124 in Siglec-7, respectively). This observation was also confirmed by mutation studies, where complete abrogation of binding was observed, when Arg97 was mutated to Ala97.[73] In the Sialoadhesin-sialoside complex, a further contribution to the overall binding affinity is related to hydrogen bonding of 5-NH to the backbone carbonyl of Ser103, the interaction of the 8-hydroxy with NH of Leu107 and the formation of a hydrogen bond of the 9-hydroxy to the backbone carbonyl of Leu107. Moreover, Trp106 establishes a lipophilic contact with C-9. All these interactions were also found in the complex of Siglec-7 with DSLc4. It is noteworthy that Arg97, Trp2 and Trp106 are conserved in all Siglecs.

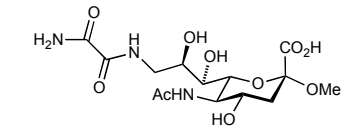


Sialoadhesin		Siglec-7	
Arg97	COO	Arg124	COO
Arg105	N5	Lys131	N5
Leu107	O8	Asn133	O8
Leu107	O9	Lys135	O9
Wat	O9	Asn133	O9
Ser103	O4	Tyr26	O5
Wat	O7		

Figure 6. 2D representation of α2,3-sialyllactose bound to Sialoadhesin.[71] Important interactions for sialic acid binding are indicated. Conserved interactions within the Siglec-family are highlighted in yellow.

Differences in the binding site of Siglec-1 and Siglec-7 were observed with regard to the hydrophobic cleft when substituents are introduced in the 9-position (*e.g.* Figure 7). Additionally, in the case of Sialoadhesin, hydrophobic interactions are established between Trp2 and the N*H*Ac residue in 5-position[8], an interaction which is not observed with Siglec-7. Also the impact of the 4- and 7-hydroxy to the binding affinity seems to be different among the Siglecs.





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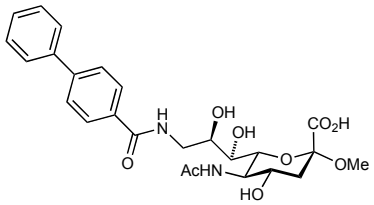

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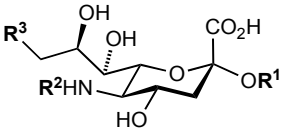
Figure 7. left: Siglec-7 in complex with Meα-9-oxamido-Neu5Ac (**1**).[10] The oxamido-substituent was reported to form hydrogen-bonds with Lys131. right: Sialoadhesin co-crystallized with 9-biphenyl-Neu5Ac (**2**).[7] The biphenyl is nicely aligned within a hydrophobic pocket. Overlay with the oxamido-compound reveals a steric clash with Val109 as the reason for non-binding of **1** to Sialoadhesin.

Amongst others, Sialoadhesin was co-crystallized with 9-naphtyl α-methyl sialoside, providing further insight into the binding mode of siglec-sialoside interactions.[7] Derivatives modified in the 5- and 9-position show identical binding modes for the sialic acid core. Additional hydrophobic interactions of 5-N*H*Ac with Trp2 and the aromatic carboxamide in the 9-position with the hydrophobic groove formed by Val109, Tyr44, Leu107, Ser45 and Asn95, respectively, led to improved affinities.[7] As Sialoadhesin is most closely related to the yet not crystallized MAG and CD22, homology models were generated on the basis of these crystallographic data.

1.4 Synthetic ligands for Siglecs.

Sialoadhesin. Interest in targeting Sialoadhesin is evolving, although up to now only a limited number of antagonists have been reported (Figure 8).[7,8,68,71,74] Sialoadhesin was co-crystallized with either the trisaccharide 3'-sialyllactose[71] or synthetic sialosides, modified in 2-, 5- and 9-position.[7,8] Detailed studies of the required functional groups for binding revealed that beside the carboxy group the hydrogen bonds of 8- and 9-OH are crucial. Modifications where these hydrogen-bond donors were abolished, led to drastically decreased binding affinities or even complete loss of affinity.[68] In contrast, 4- and 7-deoxy neuraminic acid were tolerated without drastically affecting affinity.[75] As analysis of sialyllactose binding to Sialoadhesin revealed that the main contribution results from the sialic acid moiety[71] and that the replacement of the disaccharide could be accomplished by a peptidic substitute (→**6**)[74] or benzyl aglycon (→**3**).[68]



Compound	R^1	R^2	R^3	rIP
2	Me	Ac	NH(CO) biphenyl	13
3	Bn	Ac	OH	8
4	Bn	EtAc	OH	3.4
5	Me	EtAc	OH	1.6

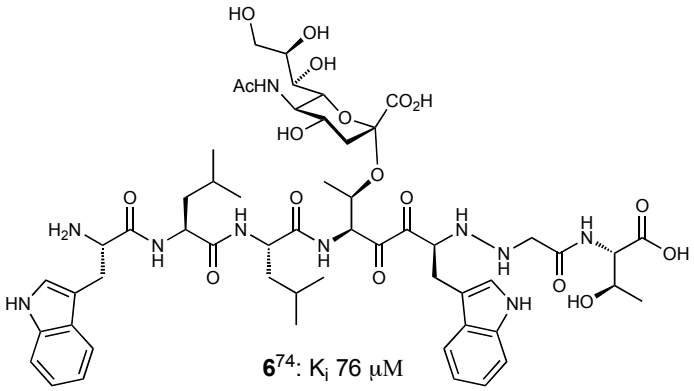


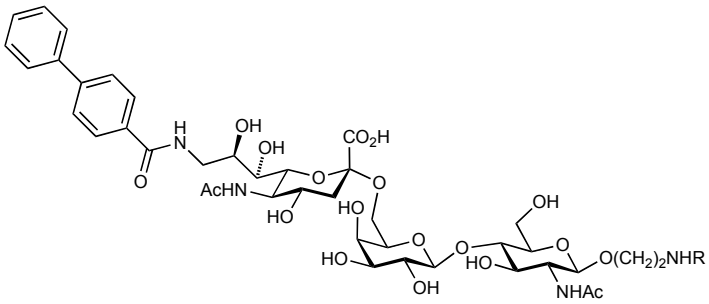
Figure 8. Synthetic ligands for Sialoadhesin. Relative inhibitory potencies (rIPs) of compounds **2**[7] and **3-5**[8] are measured against MeαNeu5Ac.

With a benzyl aglycon, binding affinity could be increased in comparison to α-methyl Neu5Ac by a factor of 8. Introduction of a benzyl carboxamide in the 9-position (→**2**) led to a further increase in binding affinity. This effect was even more pronounced in the case of biphenyl and naphtyl carboxamide[7,76]. X-ray analysis revealed favorable hydrophobic contacts, however introduction of 3,5-dinitro benzoyl or 4-acetoxy-biphenoyl was not tolerated, presumably due to a steric clash with the protein. As the acetamide in the 5-position is engaged in hydrophobic contacts with Trp2, synthetic sialosides, bearing halogenated or extended hydrophobic substituents were investigated[68]. Whereas binding affinities were only slightly increased in the case of $FH_2CC(=O)$ and $F_3CC(=O)$, propionamide and butyramide improved affinity by a factor of 2. However with *N*-benzoyl in 5-position, affinity was almost completely lost[8]. This indicates the spatial limitation of the hydrophobic groove. Currently, the best antagonists exhibit affinities in the range of 70-200 μM.

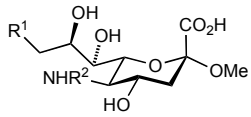
CD22 exhibits a strong preference for glycans bearing α2,6-linked sialic acids. Powell *et al*. identified the minimal structural motif required for recognition, namely Neu5Acα2,6Hex or Neu5Gcα2,6Hex, whereas Hex being Gal, Gal*N*Ac or Glc*N*Ac[33]. The minimal binding motif was further reduced to sialic acid by Rossenberg *et al.*[77] Furthermore, Kelm *et al.* showed that sialoside **2**, modified in the 9-position (Figure 7 and 9) with biphenyl carboxamide exhibits μM CD22 affinity.

Using a cellular signaling assay, specificity towards CD22 was checked and the influence on BCR inhibition was investigated. When **2** was added to B-cells, Ca^{2+} influx induced by BCR-antigen-binding was decreased and revealed that binding of **2** leads to incomplete activation of the intracellular inhibitory domain of CD22. Hence, a reduced association of CD22 with BCR leads to reduced phosphorylation[76]. These results validated CD22 as a pharmaceutical target and reveal the potential of synthetic sialosides for therapeutic applications.

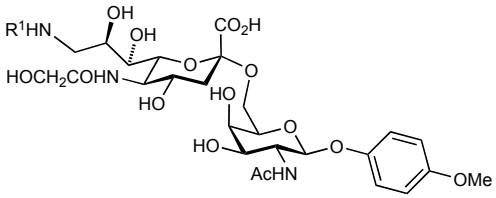
Recently, Kiso *et al*. introduced substituted biphenyl carboxamide-substituents into the 9'-position of 4-methoxy-phenyl Neu5Gcα2-6Gal,[78] and among the various tested substituents, 4-(4-hydroxyphenyl) benzamide (→**9**, Figure 9) yielded the most potent antagonist.[78] Furthermore, amines instead of amides in 9'-position were identified as affinity enhancers. Finally, for murine CD22 a replacement of the galactose moiety by a benzyl substituent was reported[79].



7 (R^1 = H)
8 (R^1 = $COCH_2$(3-NO_2-4-OH)Ph)



2 (R^1 = NH(CO)biphenyl, R^2 = Ac)
11 (R^1 = OH, R^2 = Ac)
12 (R^1 = OH, R^2 = Bz)



9 (R^1 = (CO)-(4-OH)biphenyl)
10 (R^1 = CH_2-(4-OH)biphenyl)

Compound	IC_{50} [µM]	rIC_{50}
2[76]	4.0	--
7[80]	2.2	--
8[80]	--	--
9[78]	0.4	--
10[78]	0.24	--
11[77]	--	1
12[77]	--	0.14

Figure 9. Overview on sialic acid based ligands for hCD22. Compounds **7**, **8** and **11** were also investigated as multivalent ligands. [80,77]

Several groups reported increased affinity of multivalent ligands compared to monovalent sialosides[77,81] (Figure 10). A similar observation was reported for sialosides coupled to several "supporter" such as polymers, proteins or gold nanoparticles. Collins *et al.* observed that compound **7** (9-biphenylcarboxamide-Neu5Acα2-6Galß1-4GlcNAcß-ethylamine) showed an 18-fold improved affinity compared to sialoside **2** (Figure 10).[80] Interestingly, with a multivalent presentation, *cis* interactions could be abolished without precedent removal of masking. Moreover, the introduction of a more potent sialoside into the glycan structure present on B-cells led to a "masking" that could not be overcome.[80] Therefore, the crucial point for competition with *cis* ligands is an antagonist with sufficient high potency and avidity.

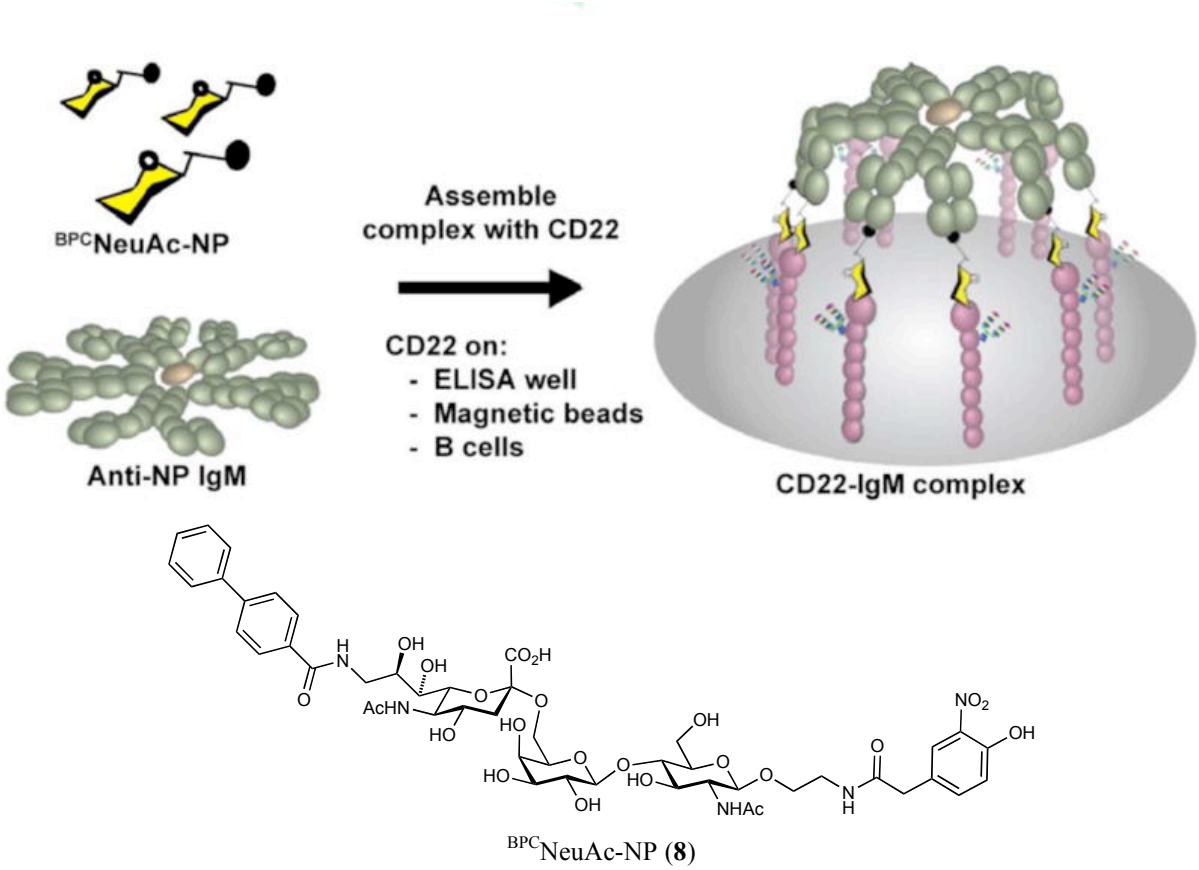
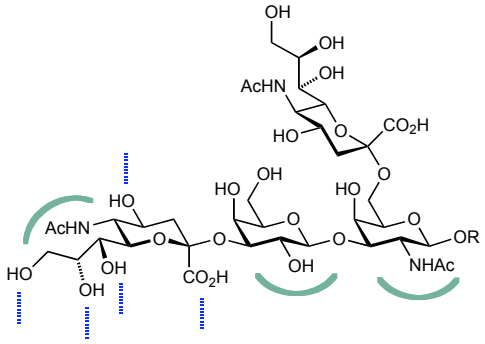


Figure 10. Multivalent display was achieved with different approaches, one example is based on self-assembly of bifunctional sialoside **8**, triggered by an antibody.[82]

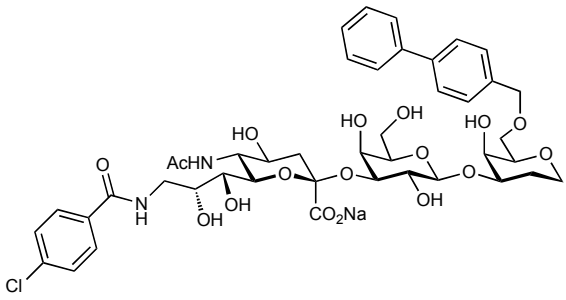
Siglec-2 (CD22) has been successfully targeted by anti-CD22 antibodies, which were applied as conjugates with different toxins.[43] The toxins were selectively transported to B-cells and internalized by antibody-triggered endocytosis. This approach of toxin-delivery was also applied for synthetic sialosides. Hence, Collins *et al.* coupled the toxin saporin to a polyacrylamide carrier, loaded with sialoside **7** (Figure 10) and observed B-cell killing.[80] Another approach is based on the design of bifunctional molecules (Figure 9), presenting a sialoside and an antigen. Upon addition of an antigen-specific antibody this led to *in situ* complex formation, providing a multivalent ligand that could overcome *cis* interaction, accompanied with an increase of the apparent K_D by a factor of 100. It is noteworthy that also a dimer binds to CD22, although higher concentrations were needed in comparison to the decavalent complex.[83]

MAG. Based on the most potent natural ligand of MAG, the ganglioside GQ1bα, antagonists with improved affinity and drug-like properties were developed.[84] Extensive structure–affinity relationship (SAR) studies led to the identification of tetrasaccharide **13**[85] as the

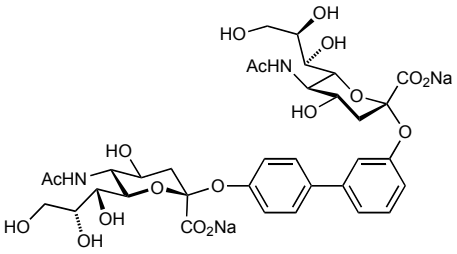
minimal binding epitope of GQ1bα.[67,86] A first generation of antagonists consisting of modified tetrasaccharides, *e.g.* **14**[20] were synthesized (Figure 11).



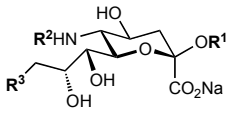
13[85] (R = OSE): K_D 180 μM
14[20] (R = Thr) : IC_{50} 300nM



15[89]: K_D 2.8 μM



16[88]



3 (R^1 = OBn, R^2 = Ac, R^3 = OH)
18 (R^1 = OMe, R^2 = FAc, R^3 = OH)
19 (R^1 = OMe, R^2 = Ac, R^3 = NHC(=O)Ph)
20 (R^1 = OBn, R^2 = Ac, R^3 = NHC(=O)*p*ClPh)

	rIP	K_D [μM]
3	10	--
18	17	--
19[87]	--	143
20[87]	--	17

Figure 11. Overview on synthetic antagonists for MAG. Salt bridge and hydrogen bonds are indicaited in blue; Green represents hydrophobic interactions with MAG.[85] Relative inhibitory potencies (rIP) for **3** and **18** were measured against MeαNeu5Ac.[68]

Further investigations of the binding event by STD NMR revealed that both, the Gal and Gal*N*Ac moiety form hydrophobic contacts with the lectin and can therefore successfully be replaced by hydrophobic moieties such as biphenyl (→**16**).[88] Furthermore, it was found that the carboxylic function of the terminal sialic acid is crucial for binding as well as the hydrogen bond capacity of 9-hydroxy and should therefore not be altered.[68,87]

In a second approach, the 2,6-linked sialic acid was substituted by hydrophilic and hydrophobic moieties. It is noteworthy that biphenyl in derivative **15** yielded a notably improved binding affinity. Combination with modifications in the 9-position of sialic acid further improved affinity to the low μM range.[89]

Finally, Kelm *et al.* and our lab reported a pivotal simplification, where sialic acid modified in 2, 5 or 9-position showed μM binding.[68,87]

1.5 Goal of the thesis.

The topic of my thesis is centered around the synthesis and biological evaluation of sialic acid based antagonists of Siglecs. Although ligands for Siglec-1, and -2 are presented, the main part is dedicated to the optimization of antagonists for Siglec-4 (MAG). Here, small-molecule antagonists modified in the 2- and 5-position were synthesized and evaluated. Furthermore, the kinetic and physicochemical properties were elucidated. For this purpose, a series of 4-modified ligands was synthesized with the attempt to influence the kinetic behavior. As found earlier in our lab, the combination of small fragments to a first site yielded highly active antagonists[90]. Based on these results, optimization and evaluation of a series of ligands was performed. Finally, the synthesized antagonists were also tested for selectivity for Siglec-1, -2 and -4.

From the Ganglioside GQ1bα to Glycomimetic Antagonists of the Myelin-Associated Glycoprotein (MAG)

This review summarizes the progresses made in the development of MAG-antagonists.

This part was published in *CHIMIA:*
B. Ernst, O. Schwardt, S. Mesch, M. Wittwer, G. Rossato, A. Vedani, *CHIMIA* **2010**, *64*, 17-22

doi:10.2533/chimia.2010.17 Chimia 64 (2010) 17–22

From the Ganglioside GQ1bα to Glycomimetic Antagonists of the Myelin-Associated Glycoprotein (MAG)

Beat Ernst*, Oliver Schwardt, Stefanie Mesch, Matthias Wittwer, Gianluca Rossato, and Angelo Vedani

Dedicated to Professor Daniel Belluš on the occasion of his 70th birthday

Abstract: The tetrasaccharide **4**, a substructure of ganglioside GQ1bα, shows a remarkable affinity for the myelin-associated glycoprotein (MAG) and was therefore selected as starting point for a lead optimization program. In our search for structurally simplified and pharmacokinetically improved mimics of **4**, antagonists with modifications of the core disaccharide Galβ(1-3)Gal*N*Ac, as well as the terminal α(2-3)- and the internal α(2-6)-linked neuraminic acid were synthesized and tested in target-based binding assays. Compared to the reference tetrasaccharide **4**, the most potent antagonist **17** exhibits a 360-fold improved affinity. Furthermore, pharmacokinetic parameters such as stability in the cerebrospinal fluid, logD and permeation through the BBB indicate the drug-like properties of antagonist **17**.

Keywords: Docking · Hapten inhibition assay · Homology model · Gangliosides · Myelin-associated glycoprotein (MAG) · MAG antagonists · Surface plasmon resonance (Biacore)

Introduction

The injured adult mammalian central nervous system (CNS) lacks the ability for axon regeneration,[1,2] predominantly due to specific inhibitors expressed on residual myelin and on astrocytes recruited to the site of injury.[3–7] Several inhibitor proteins have been identified, one of them being the myelin-associated glycoprotein (MAG).[8] MAG is a transmembrane glycoprotein[9] belonging to the so-called Siglecs, a family of the sialic acid-binding immunoglobulin like lectins.[10,11] On the surface of neurons, MAG interacts with two classes of targets: Proteins of the family of Nogo receptors (NgR)[12,13] and brain gangliosides (GD1a and GT1b)[11,14–16] (Fig. 1). Although the relative roles of gangliosides and NgRs as MAG ligands have yet to be resolved,[8,17] in some systems, MAG inhibition is completely reversed by sialidase treatment, suggesting that MAG uses sialylated glycans as its major axonal ligands.[18] Therefore, blocking MAG with potent glycomimetic antagonists may be a valuable therapeutic approach to enhance axon regeneration.

Schnaar and coworkers[19] reported that a limited set of structurally related gangliosides like GT1b or GQ1bα (Fig. 2), known to be expressed on myelinated neurons *in vivo*, are functional ligands for MAG. Recently, the MAG-affinity of a partial structure of GQ1bα, the tetrasaccharide **2**, could clearly be correlated with its ability to reverse MAG-mediated inhibition of axonal outgrowth.[22] Since SAR studies indicate that not only the terminal, α(2-3)-linked, but also the internal, α(2-6)-linked sialic acid is essential for MAG binding, various partial structures of **1**[20] as well as sulfated analogs, *e.g.* **3**[21] were synthesized.

Design of Glycomimetics

High-affinity MAG antagonists with concurrent drug-like pharmacokinetic properties would provide a valuable tool for the investigation of the exact physiological role of MAG in the inhibitory cascade leading to the collapse of growth cones, the reason for the failure of regeneration of injuries in the CNS. Because of the shallow binding site typically present in lectins, carbohydrate ligands often exhibit only modest, *i.e.* milli- to micromolar affinities.[23] This also proved true for MAG with a 180 micromolar affinity for tetrasaccharide **4**, the binding epitope of GQ1bα (Fig. 3).[24] In addition to the therefore required improvement of affinity, pharmacokinetic issues as metabolic stability, *e.g.* sialidase stability[26] or permeation of the blood brain barrier have also to be addressed. For the *in vivo* application, it is planned to add the antagonist by infusion to the site of injury. Therefore, a prolonged stability in the cerebrospinal fluid is also required. Furthermore, to maintain the necessary minimal therapeutic concentration in the CNS, a loss of the antagonist by an active or passive transport mechanism would be detrimental.

In a first approach, we focused on a reduction of the structural complexity of GQ1bα (**1**) and, at the same time, an improvement of pharmacodynamic and pharmacokinetic properties. From various structure-affinity relationship studies (SAR),[27,28] the tetrasaccharide **4** was identified as the minimal carbohydrate epitope. Detailed binding information of epitope **4** was obtained by STD NMR experiments[24] (Fig. 3). They indicated important lipophilic interactions of the glycerol side chain of the α(2-3)-linked *N*-acetyl neuraminic acid (Neu5Ac), the β-face of the galactose moiety and the *N*-acetates of both Neu5Ac residues. In addition, the carboxylates of the two Neu5Ac moieties are involved in salt bridges and the C(9)-OH of the α(2-3)-linked Neu5Ac is forming a relevant hydrogen bond.[25] A verification of these findings by docking studies to a homology model of MAG[29,30] revealed the corresponding amino acids

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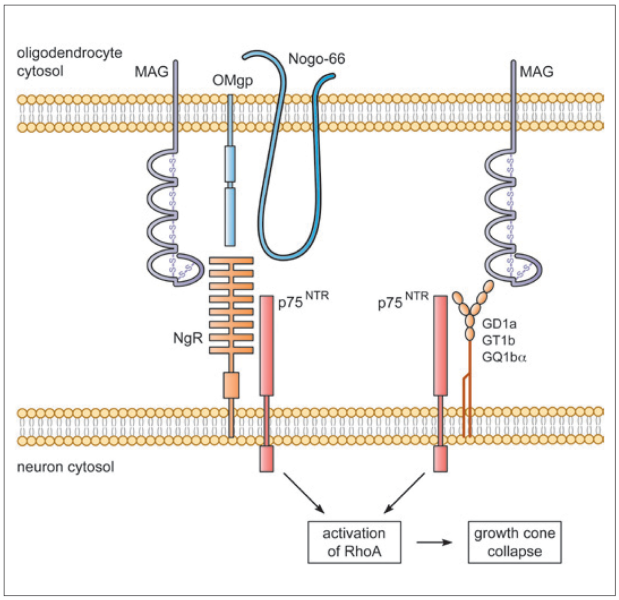


Fig. 1. Myelin-associated glycoprotein (MAG), Nogo 66 and oligodendrocyte myelin glycoprotein (OMgp) bind to the Nogo receptor (NgR). *Via* the neurotrophin receptor p75^{NTR}, the inhibitory signal is transduced into the cytosol of the neuron. MAG also binds to the gangliosides GD1a and GT1b. Again, with co-receptor p75^{NTR}, the inhibitory signal is transduced into the cytosol. Intracellularly, the small GTPase RhoA is activated, which leads to a collapse of the growth cones of the injured axon (adapted from Filbin *et al.*[5]).

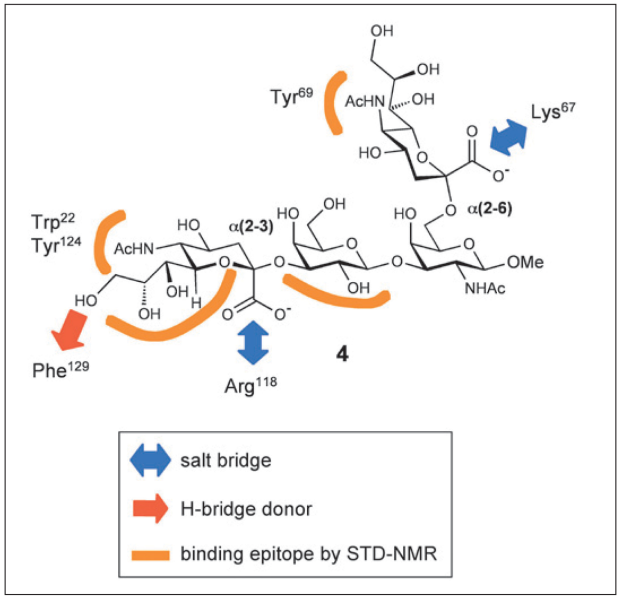


Fig. 3. Binding epitope of tetrasaccharide **4** as determined by STD NMR.[24] Besides two salt bridges by the carboxylates of the Neu5Ac moieties and an important hydrogen bond donated by the C(9)-OH of the α(2-3)-linked Neu5Ac,[25] the binding epitope involves lipophilic interactions of the glycerol side chain of the α(2-3)-linked Neu5Ac, the β-face of the galactose moiety and the *N*-acetates of the Neu5Ac residues.

forming the binding site (Figs 3 and 6). Based on this information, a rational approach for the design of MAG antagonists was envisaged.

Replacement of the Galβ(1-3)GalNAc Core

In a first approach, the Galβ(1-3) Gal*N*Ac core, establishing a lipophilic contact with MAG,[24] was replaced by biphenyl (→**5**), which acts as a linker to position the carboxylates of the two Neu5Ac moieties in the appropriate spatial orientation (Fig. 4). In addition, the biphenyl linker enables a lipophilic contact with the binding site and at the same time reduces the high polarity of the lead structure **4**. Starting from glycosyl donor **6**,[37] the building blocks **7** and **8** were synthesized, permitting the formation of the protected test compound **9** by Suzuki coupling in an excellent yield (Scheme 1). Mimic **5** was obtained after deprotection under Zemplén conditions and showed a four-fold reduction of affinity compared to tetrasaccharide **4**[36] (Table 1). For an additional structural simplification, the α(2-6)-linked Neu5Ac moiety was replaced by acetic acid leading to antagonist **10**, which showed a slightly lower affinity than **5**. To further fine-tune the spatial orientation of the carboxylates, the biphenyl linker was replaced by a 1,2,3-triazol-4-yl-phenyl moiety, a modification with practically no influence on the affinity for MAG.[38]

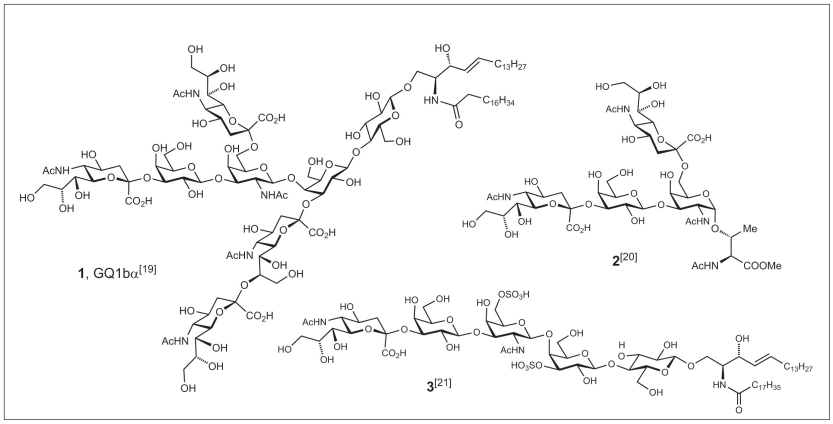


Fig. 2. GQ1bα (**1**)[19] and partial structures thereof.[20,21] Since the reported affinity data were obtained in different assay formats, they should be compared with caution. For binding of MAG-transfected COS cells to immobilized ganglioside **1** or to the disulfated GM1b analog **3**, apparent K_Ds of 38 and 7.4 pmol/well were reported.[21] In a competitive binding assay with tetrasaccharide **2**, an IC_{50} of 300 nM was determined.[20]

Lipophilic Substituents on the α(2-3)-linked Sialic Acid

A pivotal simplification of the tetrasaccharide lead structure **4** was reported by Kelm and Brossmer who modified the α(2-3)-linked sialic acid in the 2-, 5- or 9-position to obtain up to a ten-fold enhancement of affinity compared to lead **4**.[28,39,40] Further optimization of these three positions led to antagonist **17** (Scheme 2) with a 360-fold improved affinity, *i.e.* 500 nanomolar.[25,41]

Starting from the Boc-protected neuraminic acid derivative **12**,[41] **14** was obtained by deprotection with TMSCl and PhOH (→**13**) followed by acylation with fluoroacetyl chloride. Glycosylation using 2,3-difluorobenzyl alcohol (→**15**), amidation using modified Staudinger conditions[42] (→**16**) and final deprotection gave the test compound **17**.

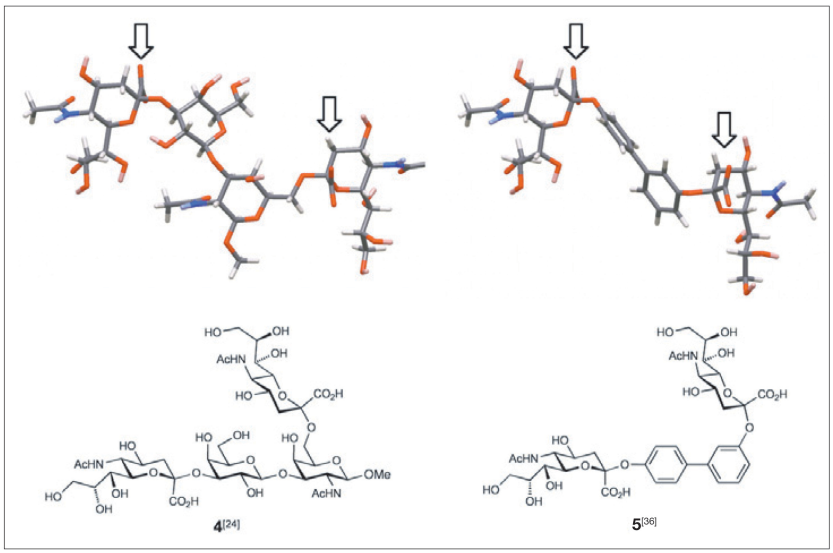
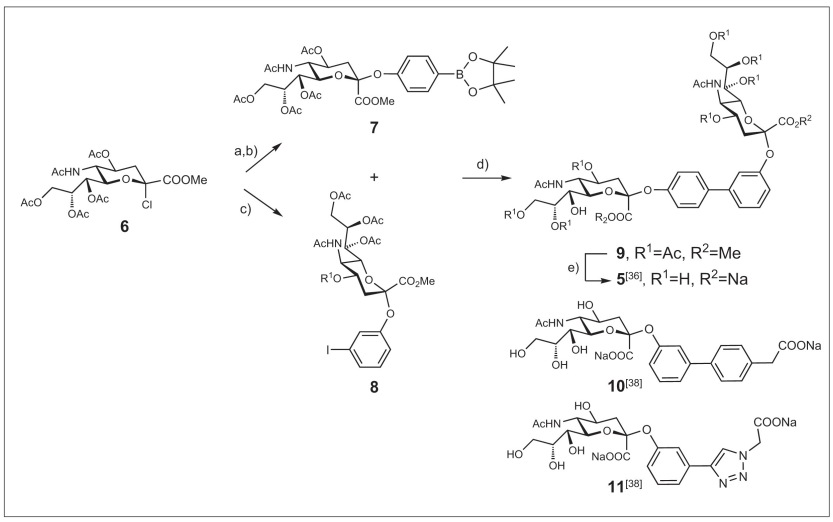


Fig. 4. Replacement of the Galβ(1-3)Gal*N*Ac core of **4** by a biphenyl (→**5**) leads to only marginal changes of the required spatial orientation of the carboxylate of the α(2-6)-linked Neu5Ac, but results in a four-fold reduction in affinity.[36]



Scheme 1. a) 4-Bromophenol, $BnNEt_3Br$, aq. NaOH/DCM, 40 °C, 2.5 h, 56%; b) bis(picanolato)diborane, KOAc, $PdCl_2$(dppf), dppf, dioxane, MW 120 °C, 45 min, 85%; c) 3-iodophenol, $BnNEt_3Br$, aq. NaOH/DCM, 40 °C, 2.5 h, 46%; d) Ag_2CO_3, $Pd(PPh_3)_4$, dioxane, MW 120 °C, 7 h, 77%; e) i. NaOMe, MeOH, r.t., 17 h, ii. aq. NaOH, r.t., 6 h, iii. Dowex 50 × 8 (Na^+), 56%.

Lipophilic and Hydrophilic Replacements of the α(2-6)-linked Sialic Acid

Since the carboxylate of the α(2-6)-linked Neu5Ac in tetrasaccharide **4** forms a salt bridge with Lys67 and the *N*-acetate a lipophilic contact with Tyr69 (Figs 3 and 6A), hydrophilic as well as lipophilic substitutes were explored. A replacement by lactic acid (→**22**) or biphenylmethyl (→**23**) yielded affinities in the range of tetrasaccharide **4** (Scheme 3). Combined with the most successful modification of the 9-position of the α(2-3)-linked Neu5Ac (Scheme 2) antagonist **29** with low micromolar affinity could be identified (Scheme 4).[30]

Biological Evaluation

Determination of Affinity for MAG

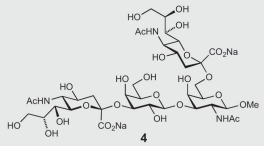
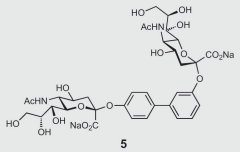
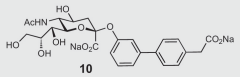
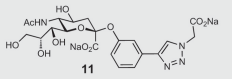
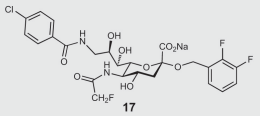
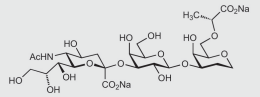
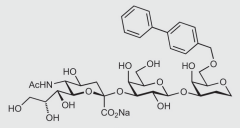
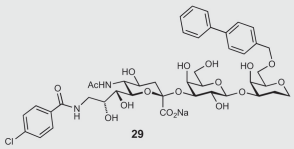
For the evaluation of the binding properties of these new MAG antagonists two assay formats were applied; a fluorescent hapten binding assay[43] and a surface plasmon resonance based biosensor (Biacore) experiment[30,41] (Fig. 5). For the hapten inhibition assay, a recombinant protein consisting of the three *N*-terminal domains of MAG and the Fc part of human IgG ($MAG_{d1\text{-}3}$-Fc) was produced by expression in CHO cells and affinity purification on protein A-agarose[43] (Fig. 5A). The relative inhibitory concentrations (rIC_{50}) of the test compounds as competitive ligands were determined in microtiter plates coated with fetuin as binding target for $MAG_{d1\text{-}3}$-Fc. By complexing the Fc-part with alkaline phosphatase-labeled anti-Fc antibodies and measuring the initial velocity of fluorescein release from fluorescein diphosphate, the amount of bound $MAG_{d1\text{-}3}$-Fc can be determined. The affinities were measured relative to the reference compound **4** (rIC_{50} of **1**, Table 1).

For the K_D determination in the Biacore assay, $MAG_{d1\text{-}3}$-Fc could not be immobilized by amine coupling, because three lysines are positioned in proximity to the carbohydrate binding site (Fig. 5B). Therefore, $MAG_{d1\text{-}3}$-Fc was immobilized on a dextran chip containing a surface of covalently bound protein A. A reference cell providing only protein A was used to compensate unspecific binding to the matrix (Figs 5C and 5D).

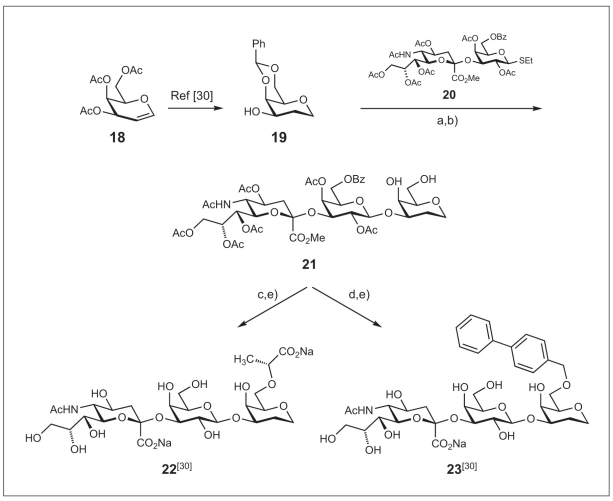
Stability in Cerebrospinal Fluid

For nerve regeneration, MAG antagonists will most likely be applied to the CNS by a local infusion. We therefore tested the stability of the fluoroacetate **17** in artificial cerebrospinal fluid (aCSF)[44] for 19 h at 37 °C and, as a control, in buffer solution. According to LC-MS analysis, more than 95% of the initial concentrations of **17** were recovered from both media, predicting a high stability in the CNS, the

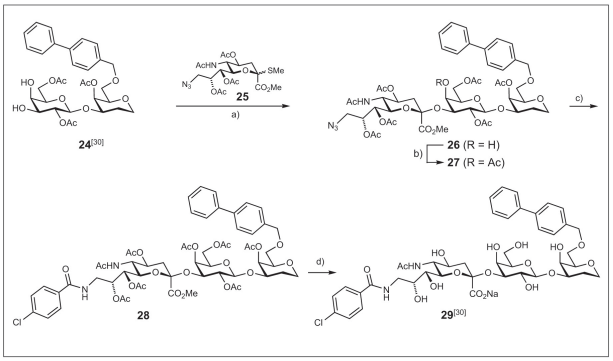
Table 1. Relative inhibitory concentrations (rIC_{50}) of MAG antagonists, K_D values of compounds **4**, **17** and **29**.

Entry	Compound	rIC_{50}[a]	K_D [μM]
1	 **4**	**1.00**	180[b]
2	 **5**	2.39	n.d.[c]
3	 **10**	4.00	n.d.[c]
4	 **11**	3.64	n.d.[c]
5	 **17**	0.0002	0.5[d]
6	 **22**	1.02	n.d.[c]
7	 **23**	0.80	n.d.[c]
8	 **29**	0.0027	2.83[d]

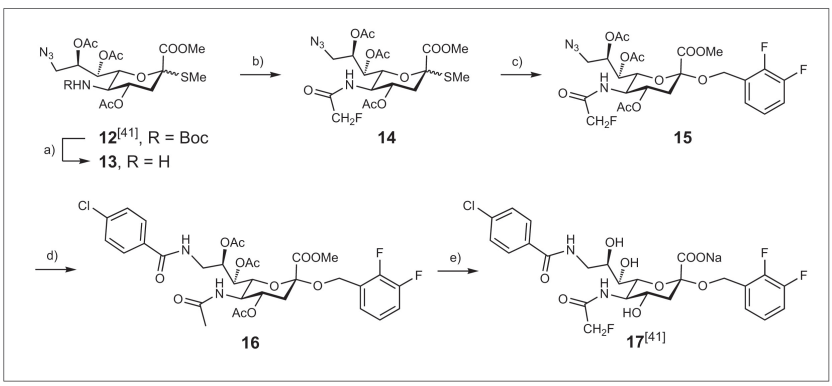
[a]rIC_{50} is the concentration when 50% of the protein are inhibited, measured relative to reference compound **4**; [b]determined by STD NMR; [c]n.d. not determined; [d]determined by surface plasmon resonance (Biacore.)



Scheme 3. a) DMTST, MS 3Å, DCM, 0 °C, 2 d, 79%; b) 80% aq. AcOH, 60 °C, 3 h, 89%; c) i. Bu_2SnO, benzene, 80 °C, 3 h, ii. ethyl (*S*)-2-*O*-(trifluoromethylsulfonyl)-lactate, CsF, DME, r.t., 1 d, 85%; d) i. biphenyl-4-carbaldehyde, cat. *p*-TsOH, MeCN, 70 °C, 4 h, ii. $Me_3N{\cdot}BH_3$, $AlCl_3$, THF, r.t., 3 h, 51%; e) NaOMe/MeOH, r.t., 1 d, then aq. NaOH, rt, 7 h, **22**: 68%, **23**: 96%.



Scheme 4. a) NIS/TfOH, MeCN, –40 °C, 61%; b) Ac_2O, pyr, 80%; c) *p*-Cl-BzCl, PPh_3, DCE, r.t., 16 h, 75%; d) NaOMe/MeOH, r.t., 3 h, then aq. NaOH, r.t., 16 h, 75%.



Scheme 2. a) 4 M PhOH, 4 M TMSCl in DCM, 70%; b) FAcCl, NEt_3, DMAP, DCM, rt, 16 h, 85%; c) 2,3-difluorobenzyl alcohol, NIS, TfOH, MeCN, α-isomer: 60%; β-isomer: 11%; d) PPh_3, *p*-Cl-BzCl, DCE, r.t., 48%; e) 10% aq. LiOH, THF/H_2O; Dowex 50 × 8 (Na^+), 30%.

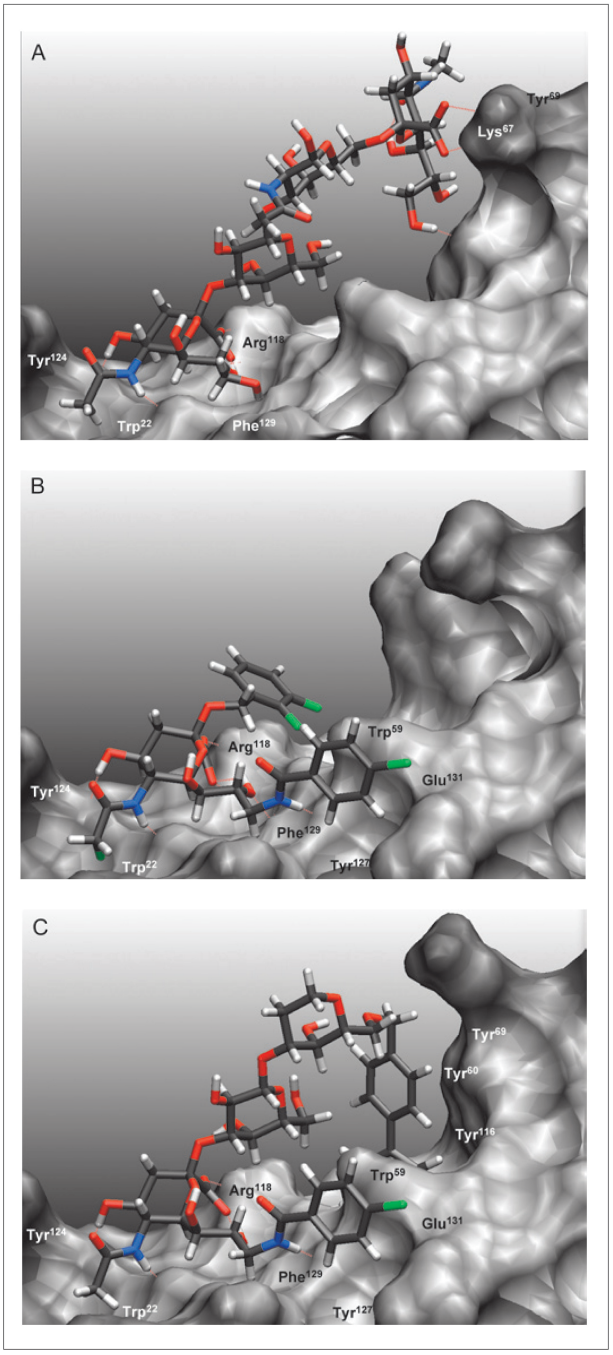


Fig. 6. The three-dimensional structures of **4**, **17** and **29** were generated using the MacroModel software[31] and optimized in aqueous solution by means of the AMBER* force field.[32] Atomic partial charges (MNDO/ESP) were then generated using MOPAC.[33] The ligands were first manually docked and the protein–ligand complex was minimized in aqueous solution and then subjected to a molecular-dynamics equilibration protocol (24 ps at 10 K, heating to 300 K during 48 ps, 1 ps = 10^{-12} s), followed by a molecular dynamic at 300 K for 4 ns performed with Desmond[34] (at 2.4 ps intervals). The structures of the trajectory along the molecular dynamic simulation have been sampled through a hierarchical cluster linkage method at 2.4 ps intervals. The images have been generated using VMD.[35] A) Tetrasaccharide **4**, a substructure of ganglioside GQ1bα, B) antagonist **29**; antagonist **17**.

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Low Molecular Weight Antagonists of the Myelin-Associated Glycoprotein: Synthesis, Docking, and Biological Evaluation

Small-molecule antagonists for MAG, based on sialic acid, were synthesized and evaluated regarding their affinity.

This part was published in the *Journal of Medicinal Chemistry:*
S. Mesch, D. Moser, D. Strasser, A. Kelm, B. Cutting, G. Rossato, A. Vedani, H. Koliwer-Brandl, M. Wittwer, S. Rabbani, O. Schwardt, S. Kelm, B. Ernst, *J. Med. Chem.* **2010**, 1597-1615.

Work performed by Stefanie Mesch:
Synthesis of MAG-Antagonists, performance and evaluation of Biacore assay.

Journal of Medicinal Chemistry Article

J. Med. Chem. **2010,** *53,* 1597–1615 **1597**
DOI: 10.1021/jm901517k

Low Molecular Weight Antagonists of the Myelin-Associated Glycoprotein: Synthesis, Docking, and Biological Evaluation

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Received August 10, 2009

The injured adult mammalian central nervous system is an inhibitory environment for axon regeneration due to specific inhibitors, among them the myelin-associated glycoprotein (MAG), a member of the Siglec family (sialic-acid binding immunoglobulin-like lectin). In earlier studies, we identified the lead structure **5**, which shows a 250-fold improved in vitro affinity for MAG compared to the tetrasaccharide binding epitope of GQ1bα (**1**), the best physiological MAG ligand described so far. By modifying the 2- and 5-position, the affinity of **5** could be further improved to the nanomolar range (→**19a**). Docking studies to a homology model of MAG allowed the rationalization of the experimental binding properties. Finally, pharmacokinetic parameters (stability in the cerebrospinal fluid, logD and permeation through the BBB) indicate the drug-like properties of the high-affinity antagonist **19a**.

Introduction

The injured adult mammalian central nervous system (CNS) lacks the ability for axon regeneration,[1,2] predominantly due to specific inhibitors expressed on residual myelin and on astrocytes recruited to the site of injury.[3−7] Several inhibitor proteins have been identified, one of them being the myelin-associated glycoprotein (MAG).[8] MAG is a transmembrane glycoprotein[9] belonging to the family of the sialic acid-binding immunoglobulin like lectins, the so-called Siglecs.[10,11] On the surface of neurons, MAG interacts with two classes of targets: proteins of the family of Nogo receptors (NgR[a])[12,13] and the gangliosides GD1a and GT1b.[11,14−16] Although the relative roles of gangliosides and NgRs as MAG ligands have yet to be resolved,[8,17] in some systems, MAG inhibition is completely reversed by sialidase treatment, suggesting that MAG uses sialylated glycans as its major axonal ligands.[18] Therefore, blocking MAG with potent antagonists may be a valuable therapeutic approach to enhance axon regeneration.

So far, the native carbohydrate ligand with the highest affinity to MAG is the ganglioside GQ1bα (Figure 1).[19] Recently, MAG affinity of a partial structure of GQ1bα, the tetrasaccharide **1**, could clearly be correlated with its ability to reverse MAG-mediated inhibition of axonal outgrowth.[20] To reduce the structural complexity of tetrasaccharide **1** and, at the same time, improve its pharmacodynamic and pharmacokinetic properties, numerous MAG antagonists have been prepared. Because the affinity of a series of gangliosides indicated that not only the terminal Neu5Acα(2−3)Gal structure is essential for MAG binding but also the internal sialic acids, the corresponding sialylated and sulfated analogues were synthesized.[21,22] Furthermore, structural information obtained by trNOE NMR[23] and STD NMR[24] suggested that the Galβ(1−3)Gal*N*Ac core contributes to binding mainly by hydrophobic contacts. This was confirmed by the successful replacement of this disaccharide substructure by noncarbohydrate linkers.[25] In addition, the α(2−6)-linked sialic acid could also be replaced by lipophilic substituents.[22] Finally, a pivotal simplification was reported by Kelm and Brossmer when they found that sialic acid derivatives modified in the 2- (e.g., **2**),[26,27] 5- (e.g., **3**),[26,27] or 9-position[26,28] (e.g., **4**) exhibited enhanced antagonistic activity.[26] Combining the best modifications found for the 2[29]- and 9-position[30] in one molecule led to antagonists, e.g., **5**, with affinities in the low micromolar range.[30]

In this communication, we report a small library of MAG antagonists based on lead compound **5**. The binding properties, evaluated by a hapten binding assay, surface plasmon resonance (SPR), and competitive NMR experiments were rationalized by docking studies to a homology model of MAG. Finally, according to pharmacokinetic parameters, e.g., stability in the cerebrospinal fluid, the drug-likeness of the identified high-affinity antagonists could be demonstrated.

Results and Discussion

The sialic acid derivatives reported by Kelm and Brossmer[26] and our group[29,30] exhibit MAG affinities in the low μM range. An example is the neuraminic acid (Neu5Ac) derivative

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[a] BBB, blood−brain barrier; CHO, Chinese hamster ovary; ClAc, 2-chloroacetyl; DCE, 1,2-dichloroethane; DCM, dichloromethane; DMAP, 4-dimethylamino-pyridine; DMF, *N*,*N*-dimethylformamide; FAc, 2-fluoroacetyl; Gal, galactose; Gal*N*Ac, *N*-acetylgalactosamine; IgG, immunoglobulin G; K_D, dissociation constant; MS, molecular sieve; Neu5Ac, *N*-acetylneuraminic acid; NgR, Nogo receptor; NIS, *N*-iodosuccinimide; NMR, nuclear magnetic resonance; nosyl, 2-nitrobenzylsulfonyl; PAMPA, parallel artificial membrane permeation assay; PDB, Protein Data Bank; *i*PrOH, 2-propanol; RP, reversed phase; STD NMR, saturation transfer difference NMR; TfOH, trifluoroacetic acid; THF, tetrahydrofurane; TMS, trimethylsilyl; trNOE, transfer nuclear Overhauser enhancement; *p*-Ts, *p*-tolylsulfonyl.

Published on Web 01/22/2010

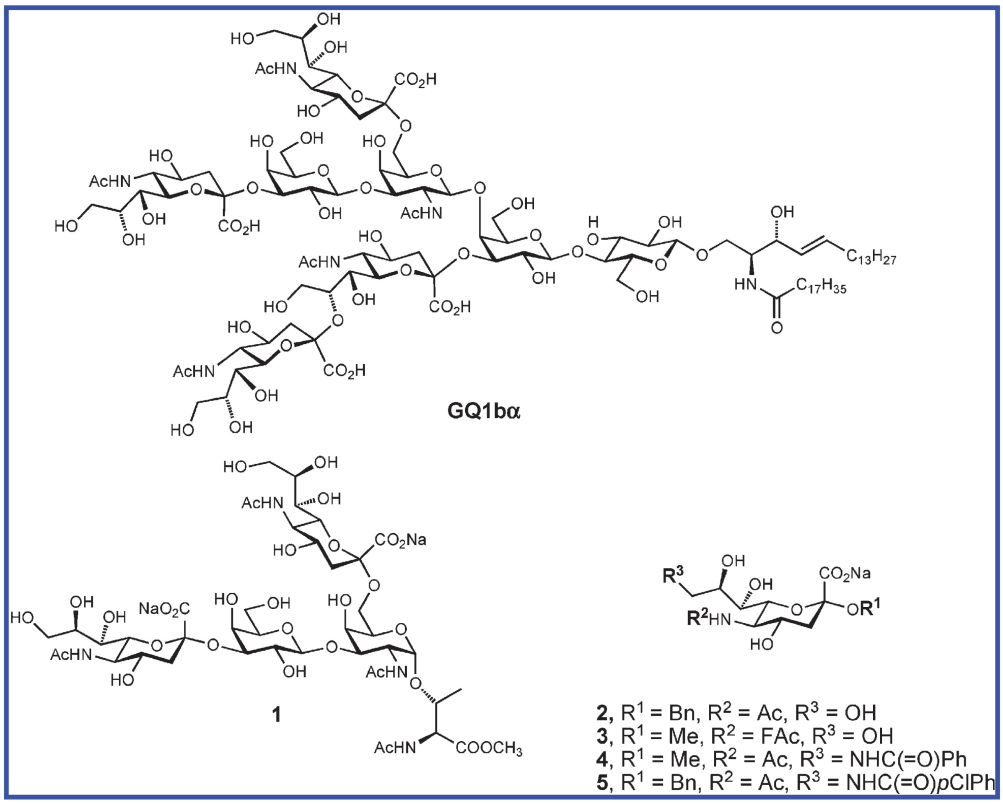


Figure 1. MAG antagonists; GQ1bα, the partial structure **1**,[19,20] and sialic acid derivatives **2**−**5**.[26,30]

5[30] with a K_D of 17 μM. Because a broad optimization effort for the 9-position had led to the identification of *p*-chlorobenzamide as the best substitutent,[30] this position was not further investigated. The changeover from a methyl substituent in the 2-position to a benzyl group was rewarded by a 10-fold gain in affinity.[26] This effect can be rationalized by the results of STD-NMR investigations,[23] indicating a hydrophobic interaction of the α-face of D-galactose (see **1** in Figure 1) with MAG. When the hydrophobic contact was further extended by replacing the benzyl group with phenoxybenzyl or biphenyl substituents, only marginally improved affinities were detected.[29] Because the sheer enlargement of the hydrophobic group did not exhibit improved affinities, the electron density of the aglycone was altered in a next step. Finally, with the substituents in the 2- and 9-position set, a further optimization of the acyl group in the 5-position was conducted.[26,27]

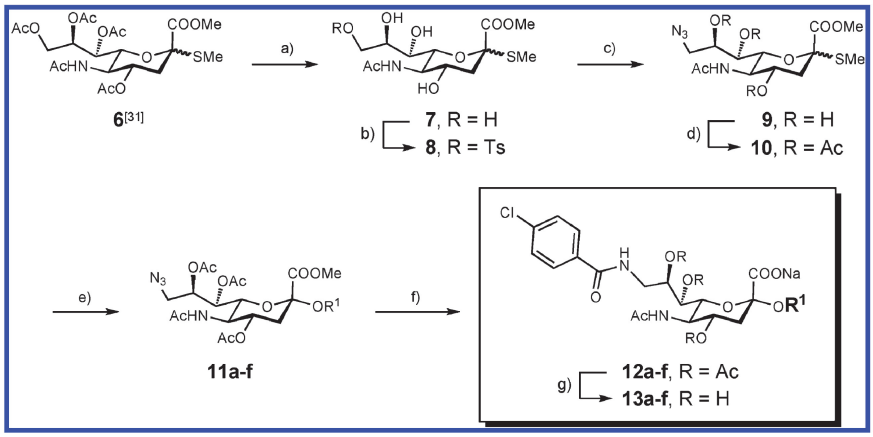
Synthesis of Sialosides 13a−f. Starting from Neu5Ac, thioglycoside **6** was synthesized according to a reported procedure.[31] After deprotection by Zemplén conditions (→**7**), the hydroxy group in the 9-position was selectively tosylated (→**8**).[32] Substitution of the tosylate using sodium azide and 15-crown-5 in DMF (→**9**)[33] followed by acetylation yielded the sialyl donor **10**. For the introduction of the aglycone, **10** was then reacted with various benzyl alcohols (see Table 1, entries 2−9) in the presence of the promotors NIS/TfOH.[34] The sialosides **11a−f** were obtained as separable anomeric mixtures (α/β = 7/1 to 9/1). Amidation with *p*-chlorobenzoylchloride under modified Staudinger conditions[35] (→**12a−f**) and final deprotection gave the amides **13a−f** in good yields (Scheme 1, Table 1).

Synthesis of Sialosides 19a−g. As reported earlier, halogenation of the *N*-acetyl group at the 5-position increases the binding affinity toward MAG by a factor of 10−20.[26] Therefore, both the *N*-fluoroacetyl and *N*-chloroacetyl derivatives **19a** and **19b** were prepared. As sulfonamides adapt a different geometry[36] compared to the corresponding amides, **19c** and **19d** allow a further exploration of the binding site. Finally, the cyclopropyl and cyclobutyl derivatives **19f** and **19g** were synthesized in order to explore the possibility for extended hydrophobic contacts.

For the cleavage of the *N*-acetate group, **10** was treated with hydrazine in the presence of $(Boc)_2O$[37,38] and subsequently reacetylated (→**14**). Deprotection with TMSCl and PhOH[39] (→**15**), followed by acylation with carboxylic or sulfonic acid derivatives, yielded **16a−d**. Glycosylation using 2,3-difluorobenzyl alcohol (→**17a−d**), amidation using modified Staudinger conditions[35] (→**18a−d**) and final deprotection gave the test compounds **19a−d**. When methanolic NaOH was used in the final deprotection step of **18b**, a nucleophilic substitution occurred, leading to a 1:1 mixture of the desired **19b** and the methoxyacetamide derivative **19e** (Scheme 2).

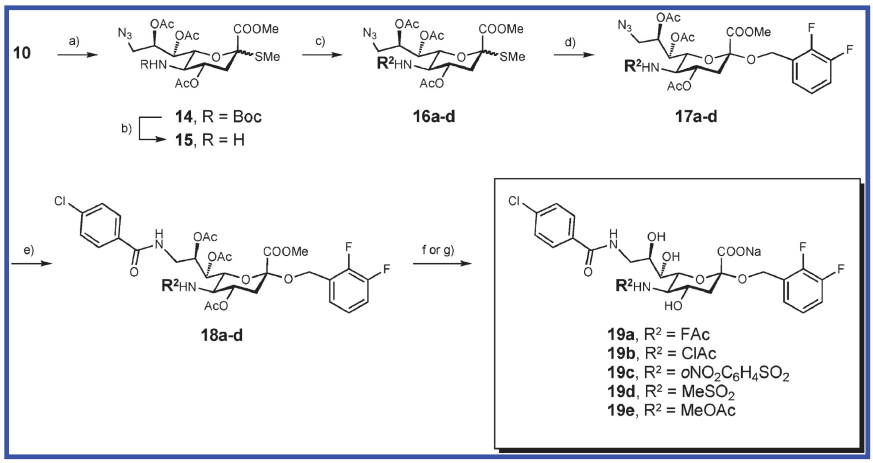
For the synthesis of the two remaining test compounds **19f** and **19g**, an analogue approach starting from the thiosialoside **10** was accomplished. However, in contrast to the synthesis of **19a−e**, a different sequence of the modifications conducted at the 2-, 5-, and 9-position was performed (Scheme 3). Cleavage of the *N*-acetate (→**14**) and amidation under modified Staudinger conditions yielded compound **20**. Next, *N*-deprotection followed by *N*-acylation (→**22f** and **22g**) and benzylation (→**23f** and **23g**) yielded, after final

Scheme 1[a]



[a] (a) NaOMe, MeOH (61%); (b) *p*-TsCl, pyridine (66%); (c) NaN_3, 15-crown-5, DMF (78%); (d) Ac_2O, DMAP, pyridine (73%); (e) R^1OH, NIS, TfOH, MeCN (**11a**−**f**, α-isomers: 54−76%; *β*-isomers: 8−11%); (f) PPh_3, *p*-chlorobenzoylchloride, DCE, rt (**12a**−**f**, 45−60%); (g) 10% aq NaOH; Dowex 50 × 8, Na^+ form (**13a**−**f**, 39−70%).

Scheme 2[a]



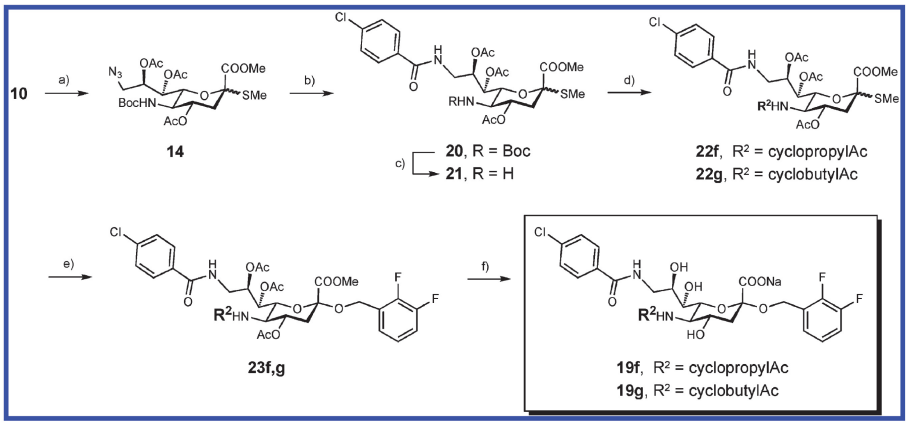
[a] (a) (i) Boc_2O, DMAP, THF, 60 °C, 4 h, (ii) $N_2H_4 \cdot H_2O$, MeOH, rt, 24 h, (iii) Ac_2O, pyridine (76%); (b) 4 M PhOH, 4 M TMSCl in abs DCM (70%);[39] (c) acylation agent, NEt_3, DMAP, abs DCM, rt, 4 h or $[ClCH_2C(=O)]_2O$, NEt_3, dioxane/H_2O, rt (**16a**−**d**, 66−85%); (d) 2,3-difluorobenzyl alcohol, NIS, TfOH, MeCN (**17a**−**d**, α-isomers: 56−68%; *β*-isomers: 8−11%); (e) PPh_3, *p*-chlorobenzoylchloride, DCE, rt (**18a**−**d**, 48−58%); (f) 10% aq LiOH, THF/H_2O; Dowex 50 × 8, Na^+ form (**19a**−**d**, 30−60%); (g) **18b**, 10% aq NaOH; Dowex 50 × 8, Na^+ form (**19b**, 21%, **19e**, 19%).

deprotection, the desired cycloalkylacetic acid derivatives **19f** and **19g** in excellent overall yields.

Biological Evaluation. For the evaluation of the binding properties of the sialosides **13a**−**f** and **19a**−**g**, two previously reported assay formats were applied: a fluorescent hapten binding assay[40] and a SPR based biosensor (Biacore) experiment[22] (Table 1). For the hapten inhibition assay, a recombinant protein consisting of the three *N*-terminal domains of MAG and the Fc part of human IgG (MAG_{d1-3}-Fc) was produced by expression in CHO cells and affinity purification on protein A-agarose.[40] The relative inhibitory concentrations (rIC_{50}) of the test compounds as competitive ligands were determined in microtiter plates coated with fetuin as the binding target for MAG_{d1-3}-Fc. By complexing the Fc-part with alkaline phosphatase-labeled anti-Fc antibodies and measuring the initial velocity of fluorescein release from fluorescein diphosphate, the amount of bound MAG_{d1-3}-Fc could be determined. At least three independent titrations were performed for each compound tested with seven or eight concentrations in triplicates. The affinities were measured relative to the reference compound **5** (rIC_{50} of 1, Table 1, entry 2). For the K_D determination in the Biacore assay, MAG_{d1-3}-Fc was immobilized on a dextran chip containing a surface of covalently bound protein A. A reference cell providing only protein A was used to compensate unspecific binding to the matrix.

By analyzing the affinities of **13a**−**f**, a substantial increase in affinity was achieved when the aromatic aglycone was halogenated in *ortho*- and *meta*-positions (entries 8 and 9). Less effective were halogens in the *para*-position (entries 4 and 5). In addition, with fluorine instead of chlorine, consistently slightly higher affinities (entries 4 and 8 vs entries 5 and 9) were obtained. With the symmetric pentafluoro benzyl group in **13c** (entry 6), an increase in affinity was expected, caused by an improved preorganization of the aglycone in the bioactive conformation. The observed

Scheme 3[a]



[a] (a) (i) Boc_2O, DMAP, THF, 60 °C, 4 h, (ii) $N_2H_4 \cdot H_2O$, MeOH, rt, 24 h, (iii) Ac_2O, pyridine (76%); (b) PPh_3, *p*-chlorobenzoylchloride, DCE, rt (48%); (c) 4 M PhOH, 4 M TMSCl in abs DCM (63%);[39] (d) R^2COCl, NEt_3, DMAP, abs DCM, rt, 4 h (**22f**, 75%, **22g**, 39%); (e) 2,3-difluorobenzyl alcohol, NIS, TfOH, MeCN (**23f**, α: 73%, *β*: 8%; **23g**, α: 72%, *β*: 8%); (f) 10% aq LiOH, THF/H_2O; Dowex 50 × 8, Na^+ form (**19f**, 44%; **19g**, 50%).

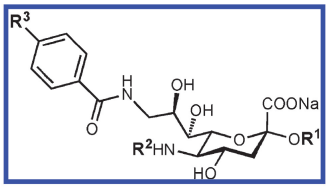
diminished affinity, i.e., a loss of a factor 3 compared to **13f**, may be the result of steric hindrance. Finally, when a 2-naphthylmethyl substituent was introduced (**13d**), a slight improvement of affinity compared to the benzyl substituent as present in **5** was obtained, presumably due to a favorable $\pi-\pi$ interaction of the extended aromatic system. In a next step, substituent R^2 was optimized based on the so far most active antagonist **13f**. Kelm and Brossmer have demonstrated that halogenated acetamides in the 5-position of sialic acid derivatives strongly improve binding of MAG antagonists.[26] When this observation was applied to our series, the nanomolar fluoroacetamide **19a** (entry 10) was obtained. For chloroacetamide derivative **19b** (entry 11), the effect was less pronounced. Interestingly, an equally potent antagonist was achieved with the phenylsulfone substituent (**19c**, entry 12), while sulfone **19d** (entry 13) suffers from a drastic loss in activity. This is quite unexpected because an increase in the size of the acyl substituent was reported to lead to a reduction in affinity,[27] an observation that was confirmed by compounds **19e**−**g** (entries 14−16).

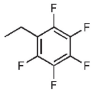
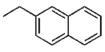
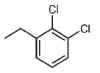
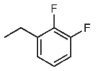
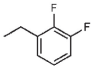
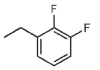
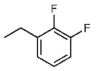
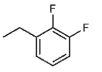
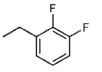
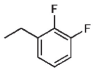
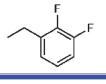
Stability in Cerebrospinal Fluid. For nerve regeneration, MAG antagonists will most likely be applied to the CNS by a local infusion. We therefore tested the stability of the fluoroacetate **19a** and the corresponding acetate **13f** in artificial cerebrospinal fluid (aCSF)[41] and, as a control, in buffer solution for 19 h at 37 °C. According to LC-MS analysis, more than 95% of the initial concentrations of both antagonists were recovered from both media, predicting a high stability in the CNS, the target compartment of an in vivo application. Furthermore, $\log D_{\text{octanol/water}}$ values from −0.27 to 0.87 (see Table 1) might be beneficial for an intrathecal application because these distribution coefficients suggest a loss from the CNS compartment by a passive transport mechanism to be unlikely. This hypothesis is further supported by the results of the BBB-PAMPA assay showing log P_e values for **13f** and **19a** in the range of −10. For values below −5.7,[42] no passive permeation through the BBB is expected.

Surface Plasmon Resonance (SPR). The interaction between MAG and MAG antagonists was analyzed by SPR experiments.[43−45] For this purpose, MAG_{d1-3}-Fc was immobilized on protein A, which on its part was covalently linked to a carboxymethyl dextrane surface of the chip. Whereas earlier Biacore investigations with MAG antagonists produced traditional sensorgrams,[22] consistently negative sensorgrams, i.e., a net decrease in resonance units, were obtained with the compound series reported in Table 1. As an example, the sensorgram of **13f** is shown in Figure 2a. When fitted to a binding isotherm, these negative sensorgrams appear to clearly result from specific receptor−ligand interactions. To elucidate the origin of this unusual result, numerous factors such as the buffer capacity, the ion strength of the buffer, type, and matrix of the sensor chip as well as the applied type of immobilization of MAG_{d1-3}-Fc were analyzed.

As a result of ionizable functional groups of the analytes, pH variabilities could occur, a phenomenon previously reported by Mannen et al.[46] To avoid this effect, a sufficient buffer capacity, 50 mM instead of 10 mM HEPES, was applied. Furthermore, to exclude ionic repulsion effects, measurements were carried out at increased salt concentrations (150−500 mM NaCl). In addition, the effect of potential nonspecific binding to the dextran matrix or to protein A was analyzed by adding either dextran (1 mg/mL) to the buffer system or by conducting the experiment on a regenerated protein A surface. Because all these modifications of assay parameters did not provide an indication for the cause of the negative sensorgrams, different dextran biosensor chips, varying in carboxylate density (CM5 vs CM4), were analyzed as well. Although the reduction in signal intensity correlated well with the degree of functionalization of the chip surface, no influence on the sign of the sensorgrams could be detected (see Table S1 in Supporting Information). A further explanation for the negative sensorgrams could be a ligand-induced conformational change of the immobilized receptor leading to a decrease of its hydrodynamic radius and, as a consequence, yielding a negative refraction index.[47,48] Because the negative refraction index correlates with the analyte concentration, we mirrored the negative sensorgrams, for an example see the sensorgram of **13f** in Figure 2b, to obtain SPR-determined equilibrium dissociation constants (K_Ds, see Table 1).

Table 1. Relative Inhibitory Concentrations (rIC_{50}) Relative to Reference Compound **5**, K_D Values, and $logD_{7.3}$ of Sialosides **13a−f** and **19a−g**



Entry	Compound	R^1	R^2	R^3	rIC_{50} [a]	K_D [μM]	logD (pH 7.3)
1	**4**[26-28]	$-CH_3$	Ac	H	18.00	137 [b]	n.d.[c]
2	**5**[30]		Ac	Cl	1.00	17	n.d.[c]
3	**24**[30]		Ac	H	1.50	26	n.d.[c]
4	**13a**		Ac	Cl	1.30	15	0.36
5	**13b**		Ac	Cl	1.20	13	-0.11
6	**13c**		Ac	Cl	0.26	6.1	-0.11
7	**13d**		Ac	Cl	0.74	11.6	0.58
8	**13e**		Ac	Cl	0.50	4.3	0.53
9	**13f**		Ac	Cl	0.30	2.4	-0.27
10	**19a**			Cl	0.02	0.5	-0.26
11	**19b**			Cl	0.07	2.1	0.35
12	**19c**			Cl	0.05	1.4	0.87
13	**19d**			Cl	0.60	17	-0.17
14	**19e**			Cl	0.14	2.3	0.06
15	**19f**			Cl	0.10	4.1	0.31
16	**19g**			Cl	0.14	5.8	0.75

[a] rIC_{50} is the concentration when 50% of the protein are inhibited, measured relative to reference compound **5**. [b] The affinity of compound **4** was measured using different protein batches, resulting in K_Ds of 137 μM and 105 μM (Table 2). All affinity data given in Table 1 were obtained with the protein batch showing a K_D of 137 μM for compound **4**; [c] n.d. not determined

To justify this procedure, we analyzed whether K_Ds obtained by mirroring the negative sensorgrams can be correlated with rIC_{50} values determined by the fluorescent hapten binding assay[40] (Figure 3). K_Ds from previously reported compounds[25,30] (see Table S2, Supporting Information), which exhibit much higher molecular weights and therefore

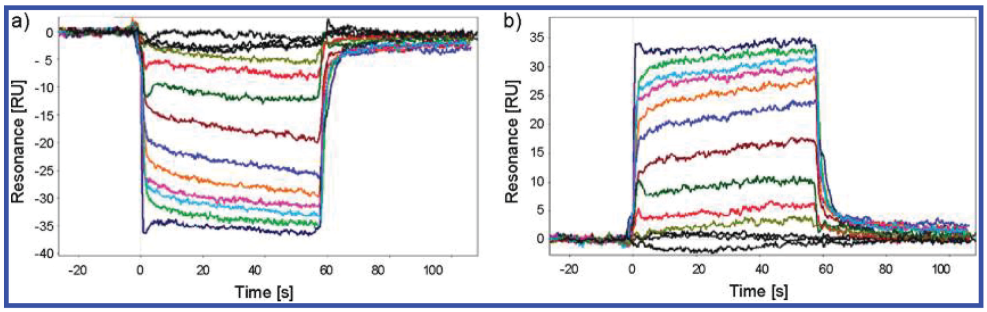


Figure 2. (a) Biacore sensorgrams for **13f** after subtraction of the reference; (b) mirrored sensorgrams of **13f**.

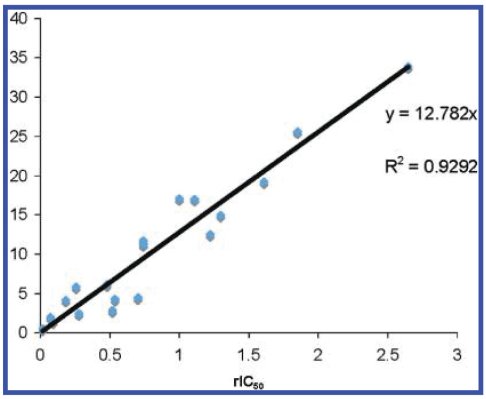


Figure 3. Correlation of K_D values obtained by SPR measurements with rIC_{50} values determined in the competitive inhibition assay;[40] Compounds **5**, **13a**−**f**, **19a**−**g**, and the compounds **25**−**29** from Table S2 (Supporting Information) were included.

generate positive sensorgrams, were also included in this correlation. The obtained correlation factor R^2 of 0.93 clearly suggests that the mirroring procedure does not falsify the binding information.

Determination of Relative Affinities by NMR. A further argument for the acceptance of the above-described mirroring of the sensorgrams was accomplished by competitive NMR experiments.[49] The approach is based on the molecular weight dependence of selective inversion recovery experiments (sT1). In the absence of binding, a selectively inverted NMR signal of a ligand requires a relatively long time to recover back to equilibrium. By contrast, if the ligand binds to a receptor, the time required to recover back to equilibrium is reduced. As a result, the binding of a ligand to a receptor is detectable through sT1 experiments. Furthermore, these sT1 experiments can be used for the ranking of the affinities of ligands relative to a reference compound.

For our purpose, the binding of antagonist **4** to MAG_{d1-3}-Fc was used as reference (K_D determined by Biacore is 137 μM), whereas compounds **13f** and **25**[22] (for the structure, see Supporting Information) were chosen as competitors because they fulfill two criteria. First, SPR experiments with both **13f** and **25** gave comparable K_D values, 2.4 and 2.8 μM, respectively, and therefore require comparable concentrations for the observation of competitive binding. Second, the observed sensorgrams of compounds **13f** and **25** are of opposite sign, negative and positive, respectively.

In a first NMR experiment, it was demonstrated that **4** binds to MAG_{d1-3}-Fc according to the large differences between selective inversion recovery of the *para*-hydrogen of the benzamide substituent in the presence or absence of MAG_{d1-3}-Fc (Figure 4a). For a quantitative evaluation of the relative affinities of **13f** and **25**, a titration curve describing the concentration dependence of the selective inversion recovery time of **4** was required (Figure 4b). The selective inversion time constants (sT1) were fit to a one-site binding model.

On the basis of the titration curve shown in Figure 4b, the determination of the relative affinities of **13f** and **25** became possible. With a NMR sample consisting of MAG_{d1-3}-Fc and compound **4** (500 μM), sT1 for the initial point of the titration curve was remeasured. The obtained sT1 of 1.03 ± 0.08 s compared to 1.05 ± 0.06 s for the first sample indicated a high degree of reproducibility. In a second step, 25 μM of either compound **13f** or **25** were added. Then, the sT1s were measured and the apparent concentration of compound **4** determined. When 25 μM of compound **13f** were added, the sT1 increased to 1.84 ± 0.06 s, indicating an apparent concentration of compound **4** of 1.36 mM. Subtracting the actual concentration of compound **4** from its apparent concentration and dividing the result by the concentration of the inhibitor yields a relative affinity of 34.4 ± 2.1 for compound **13f** with respect to the reference compound **4** with the relative affinity of 1. With the identical procedure, a relative affinity of 17.6 ± 1.0 for compound **25** with respect to compound **4** was found (Figure 5).

The competitive NMR assay demonstrates that compounds **13f** and **25** both bind to MAG_{d1-3}-Fc with an affinity more than 1 order of magnitude greater than reference compound **4**. The relative affinities displayed in Figure 5 are in good agreement with the corresponding values determined by Biacore and the hapten inhibition assay.[40] Allowing a factor of 2 in the estimation of a compound's affinity with a single technology implies that the comparison of affinities between two assay formats may differ by as much as a factor of 4. Because for any of the three assays employed the affinities of **13f** and **25** to MAG_{d1-3}-Fc are within this range, the mirroring of the negative sensorgrams for the determination of K_D values is further justified.

Determination of Enthalpic and Entropic Contributions to Binding. For the elucidation of the thermodynamic parameters of the MAG/antagonist interaction, K_Ds were measured in the Biacore assay at different temperatures. The analysis of a library of ligands containing structural modifications at the 2-, 5-, and 9-position of the neuraminic acid scaffold should allow the assignment of the enthalpic and entropic contributions to the various structural elements. The K_D values were determined at six different temperatures, starting at 5 °C and elevating the temperature by 5 °C

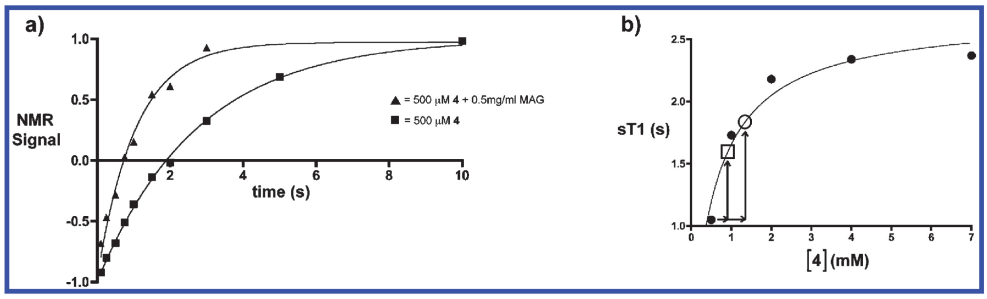


Figure 4. (a) Selective inversion recovery (sT1) of the *para*-hydrogen of the benzamide in compound **4**, either in the presence of MAG_{d1-3}-Fc (filled triangles) or in the absence of MAG_{d1-3}-Fc (filled squares). The normalized NMR signal represents the intensity of the *para*-hydrogen at a particular time divided by its intensity after 60 s of relaxation. (b) Titration of MAG_{d1-3}-Fc with compound **4**, and the observed sT1 (filled circles), observed sT1 when 500 μM of **4** were mixed with 25 μM **13f** (hollow circle), and observed sT1 when 500 μM of **4** were mixed with 25 μM **25** (hollow square). Vertical and horizontal arrows indicate the extent of attenuated relaxation of **4** when mixed with 25 μM or competitor **13f** or **25** and the apparent concentration of **4**, respectively.

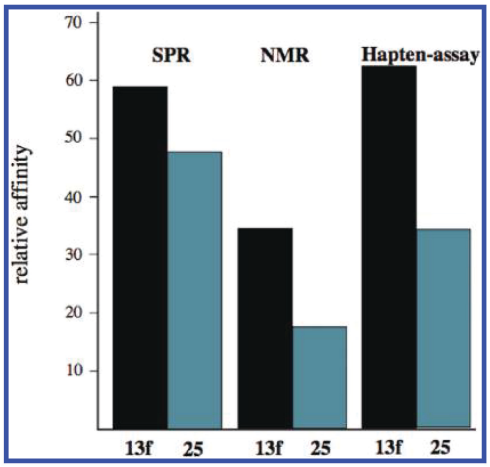


Figure 5. Affinity of compounds **13f** (black) and **25** (gray), relative to the affinity of compound **4** (= relative affinity of 1). The relative affinities are determined by NMR, Biacore, and the fluorescent hapten assay.[40]

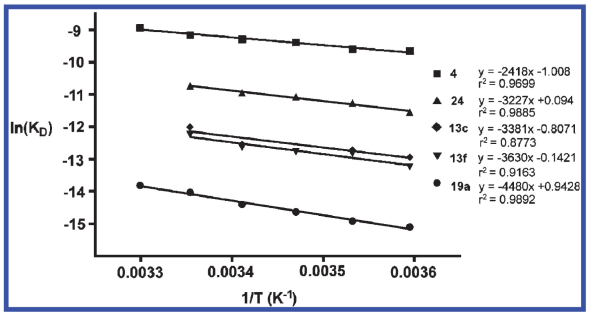


Figure 6. Van't Hoff plot. Measured data (dots) and corresponding linear fits according to eq 1.

$$\ln K_D = \frac{\Delta H}{RT} - \frac{\Delta S}{R} \qquad (1)$$

steps up to 30 °C. The values were fitted according to eq 1 (Figure 6) leading to ΔH and ΔS[50] (Table 2).

The analysis (Table 2) revealed that the improvement of the binding energies ΔG resulted mainly from enhanced binding enthalpies ΔH. The substitution of the methoxy group at position 2 in **4** by a benzyloxy group (**24**, entry 18)[30] increased binding enthalpy by more than 6 kJ/mol and at the same time caused substantial entropy costs upon binding. With the pentafluorobenzyloxy substituent (**13c**, entry 19), both ΔH and ΔS were improved. Apparently, the interaction of the reducing end substituent can be optimized with an electron-poor aromatic ring. When the 2,3-difluorinated benzyloxy substituent (**13f**, entry 20) was introduced, ΔG could be further improved, mainly by a favorable enthalpy change. On the other hand, entropy costs increased as a result of the asymmetric substitution. The binding energy of the most active compound **19a** (entry 21, *N*-acetate replaced by *N*-fluoroacetate) is mainly based on a further enthalpic improvement. Unfortunately, in this case entropy costs of more than 2 kJ/mol have to be compensated, probably due to a specific conformational orientation requested for an optimal interaction of the FAc group.

Table 2. Weighting of ΔH and ΔS with Respect to ΔG

entry	compd	ΔH [kJ/mol]	$-T^a\Delta S^*$ [kJ/mol]	ΔG [kJ/mol]	K_D [μM]
17	**4**[30]	−20.09	−2.52	−22.61	106[b]
18	**24**[30]	−26.85	0.25	−26.60	22
19	**13c**	−28.13	−1.98	−30.1	6.1
20	**13f**	−30.31	−0.20	−30.52	2.4
21	**19a**	−37.33	2.42	−34.91	0.5

[a] T = 298.13 K. [b] The affinity of compound **4** was measured using different protein batches, resulting in K_Ds of 137 μM and 106 μM. For the data given in Table 2, the protein batch showing a K_D of 106 μM for compound **4** was used.

Structure−Affinity Relationships. To interpret the binding affinities at the molecular level, we performed molecular docking studies. In the absence of a crystal structure, we used a homology model of the ligand-binding domain of MAG, which was recently applied successfully to a series of MAG antagonists (Figure 7).[22] The compounds were manually docked using the salt bridge between the carboxylate of the sialosides with Arg118 and the hydrogen bond of C(9)-NH to the backbone nitrogen of Thr128 as anchor points. Finally, the protein−ligand complexes were fully minimized in aqueous solution.

For validation purposes, 12 compounds were docked and their binding strength quantified. Because these compounds bind at the protein surface, the contribution of solvation and entropy are difficult to estimate from thermodynamic docking studies. We therefore performed a series of molecular-dynamical simulations (4.0×10^{-9} s at 300 K) to elucidate the

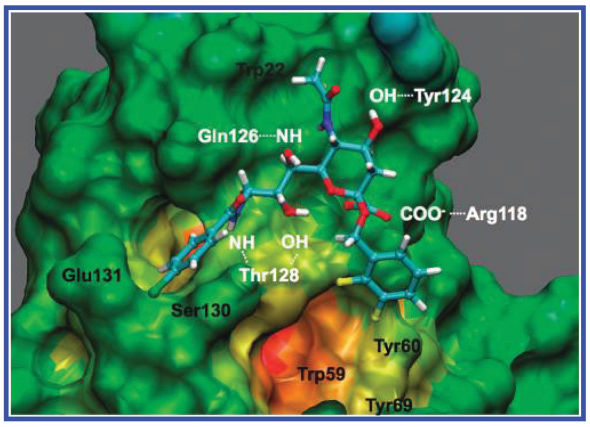


Figure 7. Homology model of MAG[22] complexed with **19a** (most active compound of the series). Amino acids in white are forming hydrogen bonds and amino acids in black contribute to hydrophobic pockets. The salt bridge formed by the carboxylate group of **19a** with Arg118 and a hydrogen bond by C(9)-NH and the backbone nitrogen of Thr128 were used as anchor points for the docking. Hydrophobic interactions are established by the fluoroacetamido group and the side chains of Trp22 and Tyr124, the *p*-chlorobenzamide, and the side chains of Ser130 and Glu131, and the reducing end substituent, the 2,3-difluorobenzyl and the side chains of Trp59, Tyr60, and Tyr69, lining the main hydrophobic pocket. The image has been generated using VMD.[51]

kinetic aspects of binding and to quantify the contribution of hydrogen bonding over time. Details are given in the Supporting Information.

Upon analyzing the docking studies, the binding affinity could be associated to hydrophilic as well as hydrophobic interactions. The most important contribution is the salt bridge between the carboxylic acid and Arg118.[26,52,53] Additionally, hydrogen-bond formations between 5-NH and the backbone carbonyl of Gln126, the carboxylate and the OH of Thr128, 8-OH and the backbone NH of Thr128 and 9-NH and the backbone carbonyl of Thr128 are observed. This latter finding is in good agreement with previous studies, where the abolishment of a hydrogen bond donor at position 9 resulted in a reduced binding affinity.[26] A considerable contribution to the binding affinity results from hydrophobic interactions. Thus, the *p*-chlorobenzamide is shown to point into a hydrophobic pocket, built by Ser130 and Glu131. A second hydrophobic pocket, which hosts the aglycone substituents, is defined by the side chains of Trp59, Tyr60, and Tyr69. With respect to different substitution patterns, the dichloro compound **13e** (Table 1, entry 8) shows a 4-fold and the difluoro compound **13f** (see Table 1, entry 9), a 7-fold enhancement in affinity compared to reference compound **5**, indicating a charge transfer complex with the electron rich aromatic ring of Tyr60. The only moderate improvement in binding affinity for the halogenated compounds **13a** and **13b** could be due to a steric clash of the *p*-substitutent with the protein. Compound **13c** was synthesized as a symmetric analogon of compound **13f** in order to compensate entropy loss due to the orientation of the 2,3-difluorobenzyl ring. Again, that there is no improvement of the binding affinity in comparison to compound **13f** may be the consequence of a steric clash based on the *p*-substitutent. Finally, the improved binding of **13d** might result from favorable $\pi-\pi$ interactions of the naphthalene with Tyr69.

Some of the compounds modified at the 5-position seem to undergo a favorable $\sigma-\pi$ interaction with Trp22. In case of **19a** and **19b**, we assume that the positively polarized hydrogens of the FAc or ClAc, respectively, stick favorably into the aromatic ring.[54] As fluorine is more electronegative, the polarization of the hydrogens is stronger and therefore the interaction is more favorable. For compounds **19f** and **19g**, additional hydrophobic interactions are possible, but the binding site seems to be spatially limited.[27] The reduced affinity of **19d** could be a consequence of the different bond angle for the sulfonamide substituent compared to an acetate in the same position (**13f**, entry 9), leading to different spatial requirements. Whereas methylsulfonamide **19d** shows a decrease in binding affinity, the nosyl substituent (**19c**) shows an opposite behavior. This might be due to the formation of a charge transfer complex with Trp22. To summarize, modifications at the reducing end improved binding affinity by a factor of 7 (**5**→**13f**). Combined with the best modification at the 5-position, the high affinity ligand **19a** was obtained.

Conclusion

In conclusion, the nanomolar affinity of the sialic acid derivative **19a** containing a difluorobenzyl substituent at the 2-, a fluoroacetate at the 5-, and *p*-chlorobenzamide at the 9-position clearly indicates the additivity of the beneficial effect of the various modifications. In addition, the thermodynamic analysis reveals that the improved affinity of **19a** predominantly results from an increased binding enthalpy and not from an entropy gain. The beneficial pharmacokinetic properties, e.g., a high stability in the cerebrospinal fluid, also support the drug-like properties of the newly identified MAG antagonist. However, due to the shallow binding site generally responsible for short half-lifes of carbohydrate−protein complexes,[22,55−58] it remains to be shown whether the complex formed by **19a** and MAG exhibits sufficient kinetic stability for in vivo applications.

Experimental Section

Chemistry. NMR spectra were recorded on a Bruker Avance DMX-500 (500 MHz) spectrometer. Assignment of ^{1}H and ^{13}C NMR spectra was achieved using 2D methods (COSY, HSQC, TOCSY). Chemical shifts are expressed in ppm using residual $CHCl_3$, CHD_2OD, and HDO as references. Optical rotations were measured using Perkin-Elmer polarimeters 241 and 341. MS analyses were carried out using a Waters Micromass ZQ Detector system. The spectra were recorded in positive or negative ESI mode. The HPLC/HRMS analyses were carried out using a Agilent 1100 equipped with a photodiode array detector and a Micromass QTOF I equipped with a 4 GHz digital-time converter. All target compounds exhibit a purity of ≥95%. Reactions were monitored by TLC using glass plates coated with silica gel 60 F_{254} (Merck) and visualized by using UV light and/or by charring with a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H_2SO_4). Column chromatography was performed on silica gel (Uetikon, 40−60 mesh). Methanol was dried by refluxing with sodium methoxide and distilled immediately before use. Pyridine was freshly distilled under argon over CaH_2. Dichloromethane (DCM), dichloroethane (DCE), acetonitrile (MeCN), toluene, and benzene were dried by filtration over Al_2O_3 (Fluka, type 5016 A basic). Molecular sieves (3 Å) were activated under vacuum at 500 °C for 2 h immediately before use. Compound **6** was prepared according to a published procedure.[31] HPLC chromatograms and ^{1}H NMR spectra of the target compounds can be found in the Supporting Information.

Methyl (Methyl 5-Acetamido-3,5-dideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (7). Compound **6** (217 mg, 42.0 mmol) was dissolved in dry MeOH (8.0 mL) and treated with NaOMe (1 M, 1.0 mL) for 2 h. The reaction mixture was neutralized with Amberlyst 15, filtered over a pad of celite, and the celite washed thoroughly with MeOH. The solvent was evaporated under reduced pressure, and the crude product was purified by chromatography on silica gel (1% gradient MeOH in DCM) to yield **7** as a white foam (90.0 mg, 61%). ^{1}H NMR (500 MHz, CD_3OD) δ 1.97 (dd, J = 11.5, 13.9 Hz, 1H, H-3a), 2.03 (s, 3H, SMe), 2.09 (s, 3H, NHAc), 2.47 (dd, J = 4.9, 13.9 Hz, 1H, H-3b), 3.54 (d, J = 9.4 Hz, 1H, H-7), 3.67 (dd, J = 5.6, 11.6 Hz, 1H, H-9a), 3.81−3.86 (m, 6H, H-5, H-8, H-9b, OMe). 4.11 (m, 2H, H-4, H-6). ^{13}C NMR (CD_3OD) δ 11.2 (SMe), 22.7 (NHAc), 41.2 (C-3), 53.1 (OMe), 54.1 (C-5), 65.2 (C-9), 68.3 (C-4), 70.2 (C-7), 71.2 (C-8), 72.6 (C-6), 84.6 (C-2), 170.8, 175.0 (2 CO). ESI-MS calcd for $C_{13}H_{23}NO_8S$ $[M + Na]^+$ 376.10; found m/z 376.10.

Methyl (Methyl 5-Acetamido-3,5-dideoxy-2-thio-9-tosyl-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (8). To a solution of **7** (3.20 g, 9.07 mmol) in freshly distilled pyridine (80 mL) *p*-toluenesulfonyl chloride (1.90 g, 10.0 mmol) was added at 0 °C and the mixture was stirred for 2 h at 0 °C. Afterward tosyl chloride (0.70 g, 3.68 mmol) was added and the solution stirred continuously for 16 h at 5 °C. The reaction mixture was warmed to rt, methanol (20 mL) was added and stirring continued for 30 min. After evaporation of the solvents, the crude product was purified by chromatography on silica gel (DCM:MeOH, 19:1) to give **8** as a foam (3.00 g, 66%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.79 (dd, J = 11.2, 13.4 Hz, 1H, H-3a), 1.96 (s, 3H, SMe), 1.99 (s, 3H, NHAc), 2.37 (s, 3H, CH_3), 2.74 (dd, J = 3.7, 13.2 Hz, 1H, H-3b), 3.25 (d, J = 9.5 Hz, 1H, H-6), 3.41 (d, J = 9.7 Hz, 1H, H-7), 3.66 (s, 3H, OMe), 3.98 (m, 1H, H-8), 4.10 (m, 1H, H-9a), 4.24 (m, 1H, H-9b), 6.94 (d, J = 7.2 Hz, 1H, N*H*Ac), 7.28, 7.72 (AA′, BB′ of AA′BB′, J = 8.2 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 11.9 (SMe), 21.7 (CH_3), 23.0 (NHAc), 40.4 (C-3), 52.7 (C-5), 53.7 (OMe), 67.8, 68.6, 69.1 (C-4, C-7, C-8), 72.0 (C-6), 82.4 (C-2), 128.0, 130.0, 132.4, 145.1 (6 C−Ar), 170.4, 174.2 (2 CO). ESI-MS calcd for $C_{20}H_{29}NO_{10}S_2$ $[M + Na]^+$ 530.11; found m/z 530.19.

Methyl (Methyl 5-Acetamido-9-azido-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (9). Compound **8** (160 mg, 0.32 mmol) was dissolved in dry DMF (5 mL). NaN_3 (103 mg, 1.58 mmol) and 15-crown-5 (28.6 mg, 0.13 mmol) were added successively and the reaction mixture was stirred at 60 °C for 24 h. After filtration through a pad of celite, the solvent was evaporated and the residue was purified by chromatography on silica gel (gradient 1% MeOH in DCM) to yield **9** (96.0 mg, 78%). ^{1}H NMR (500 MHz, CD_3OD) δ 1.79 (m, 1H, H-3a), 2.03 (s, 3H, SMe), 2.17 (s, 3H, NHAc), 2.77 (dd, J = 4.5, 12.8 Hz, 1H, H-3b), 3.39 (dd, J = 6.2, 12.7 Hz, 1H, H-9a), 3.48 (m, 2H, H-6, H-7), 3.57 (dd, J = 2.3, 12.7 Hz, 1H, H-9b), 3.69 (m, 1H, H-4), 3.79 (m, 1H, H-5), 3.86 (s, 3H, OMe), 3.93 (ddd, J = 2.6, 5.7, 8.5 Hz, 1H, H-8), 4.01 (m, 1H, H-6). ^{13}C NMR (CD_3OD) δ 12.0 (SMe), 22.7 (NHAc), 41.8 (C-3), 53.6 (C-5), 53.8 (OMe), 55.2 (C-9), 69.0 (C-4), 71.0 (C-7), 71.7 (C-8), 76.8 (C-6), 171.7, 175.2 (2 CO). ESI-MS calcd for $C_{13}H_{22}N_4O_7S$ $[M + Na]^+$ 401.11; found m/z 401.15.

Methyl (Methyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (10). Compound **9** (87.0 mg, 0.23 mmol) was dissolved in dry pyridine (1.0 mL) and cooled to 0 °C. After 15 min, DMAP (4.47 mg, 0.04 mmol) and Ac_2O (0.5 mL) were added successively. The reaction mixture was warmed to rt and stirred for 14 h. The solvent was evaporated and the residue purified by chromatography on silica gel (toluene/ethylacetate, 1:3) to afford **10** (85.0 mg, 73%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.81 (s, 3H, NHAc), 1.91 (m, 1H, H-3a), 1.97, 2.05, 2.10 (3 s, 9H, 3 OAc), 2.13 (s, 3H, SMe), 2.67 (dd, J = 4.4, 12.5 Hz, 1H, H-3b), 3.19 (dd, J = 5.7, 13.4 Hz, 1H, H-9a), 3.59 (m, 1H, H-9b), 3.76 (m, 4H, H-6, OMe), 4.01 (m, 1H, H-5), 4.81 (m, 1H, H-4), 4.91 (d, J = 7.7 Hz, 1H, N*H*Ac), 5.24 (m, 2H, H-7, H-8). ^{13}C NMR ($CDCl_3$) δ 12.1 (SMe), 20.9, 21.1 (3C, 3 OAc), 23.2 (NHAc), 37.8 (C-3), 49.4 (C-5), 50.6 (C-9), 53.0 (OMe), 68.1 (C-4), 69.7 (C-7), 72.3 (C-6), 74.6 (C-8), 88.4 (C-2), 170.2, 170.3, 171.0 (5C, CO). ESI-MS calcd for $C_{19}H_{28}N_4O_{10}S$ $[M + Na]^+$ 527.40; found m/z 527.21.

General Procedure for the Synthesis of Compounds 11a−f, 17a−d, 18f,g. Compound **10** (0.12 mmol) was dissolved in dry acetonitrile (2.0 mL) under argon. The alcohol (0.26 mmol) and powdered MS 3 Å (80.0 mg) were added. The mixture was stirred at rt for 1.5 h. Then the suspension was cooled to −40 °C and subsequently treated with *N*-iodosuccinimide (0.60 mmol) and triflic acid (0.06 mmol in 0.2 mL MeCN). After 30 min, the reaction mixture was warmed to −30 °C and stirring continued for 16 h. The mixture was then warmed to rt, stirred for another 2 h, and filtered through a pad of celite. The celite was washed with DCM (10 mL), and the filtrate was subsequently washed with 20% aqueous $Na_2S_2O_3$ (1 mL) and saturated aqueous $NaHCO_3$ (3 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel.

General Procedure for the Synthesis of Compounds 12a−f, 20, 18a−d. Compounds **11a−f** (**14** or **17a−d**) (0.09 mmol) and *p*-chlorobenzoyl chloride (0.36 mmol) were dissolved in dry DCE (3.0 mL) under argon. Triphenylphosphine (0.18 mmol) in dry DCE (1.5 mL) was added after 5 min, and the solution was stirred at rt for 24 h. The reaction mixture was diluted with DCM (10 mL) and washed with saturated aqueous $NaHCO_3$ (3 × 10 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by chromatography on silica gel.

General Procedure for the Deprotection of 12a−f, 18b. Compound **12a−f** (**18b**) (0.06 mmol) was dissolved in MeOH (1.5 mL) and treated with 10% aqueous NaOH (0.3 mL). The reaction mixture was stirred at rt for 3 h. Then the reaction mixture was neutralized with 7% aqueous HCl (0.2 mL). The solvent was evaporated, and the crude product was purified by chromatography on RP-18.

General Procedure of the Deprotection of 18a−d,f−g. Compound **18a−d,f−g** (0.03 mmol) was dissolved in THF/H_2O (2 mL/0.5 mL) and was reacted with LiOH (0.28 mmol). The crude product was purified on RP-18 (10% gradient MeOH in water) followed by ion exchange chromatography (Dowex-50) and P2 size exclusion chromatography to yield **19**.

Methyl (4-Chlorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (11a). Compound **10** (61.0 mg, 0.12 mmol) was reacted with 4-chlorobenzyl alcohol (38.0 mg, 0.26 mmol), *N*-iodosuccinimide (134 mg, 0.601 mmol), and triflic acid (6.00 μL, 9.00 mg, 0.06 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient *i*PrOH in petrol ether/DCM 8:4) to yield **11a** (55.0 mg, 76%) as a colorless oil. $[\alpha]^{20}_D$ −0.02 (*c* 0.28, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.90 (s, 3H, NHAc), 2.03 (m, 4H, OAc, H-3a), 2.17, 2.20 (2 s, 6H, 2 OAc), 2.65 (dd, J = 4.6, 12.9 Hz, 1H, H-3b), 3.26 (dd, J = 5.7, 13.4 Hz, 1H, H-9a), 3.55 (dd, J = 2.8, 13.4 Hz, 1H, H-9b), 3.71 (s, 3H, OMe), 4.12 (m, 2H, H-5, H-6), 4.41, 4.76 (A, B of AB, J = 12.3 Hz, 2H, CH_2Ar), 4.87 (m, 1H, H-4), 5.15 (d, J = 9.8 Hz, 1H, NH), 5.35 (m, 2H, H-7, H-8) 7.30 (m, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.8, 20.9, 21.1 (3 OAc), 23.3 (NHAc), 38.0 (C-3), 49.4 (C-5), 51.0 (C-9), 52.8 (OMe), 66.1 (CH_2Ar), 67.9 (C-7), 68.9 (C-4), 69.4 (C-8), 72.8 (C-6), 98.6 (C-2), 128.4, 129.1, 133.5, 135.7 (6 C−Ar), 168.3, 170.3, 170.3, 171.0 (5C, 5 CO). ESI-MS calcd for $C_{25}H_{31}ClN_4O_{11}$ $[M + Na]^+$ 621.17; found m/z 621.24.

Methyl (4-Fluorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (11b). Compound **10** (62.0 mg, 0.12 mmol) was reacted with 4-fluorobenzyl alcohol (38.0 mg, 0.26 mmol), *N*-iodosuccinimide (134 mg, 0.601 mmol), and triflic acid (6.00 μL, 9.00 mg, 0.06 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient *i*PrOH in petrol ether/DCM 8:4) to yield **11b** (53.0 mg, 76%) as a colorless oil. $[\alpha]^{20}_D$

−0.01, (*c* 2.6, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.89 (s, 3H, NHAc), 2.02 (m, 1H, H-3a), 2.03, 2.17, 2.20 (3 s, 9H, 3 OAc), 2.65 (dd, J = 4.5, 12.8 Hz, 1H, H-3b), 3.29 (dd, J = 5.4, 13.3 Hz, 1H, H-9a), 3.58 (dd, J = 2.0, 13.1 Hz, 1H, H-9b), 3.71 (s, 3H, OMe), 4.13 (m, 2H, H-5, H-6), 4.41, 4.76 (A, B of AB, J = 11.9 Hz, 2H, CH_2Ar), 4.85 (m, 1H, H-4), 5.35 (m, 2H, H-8, H-7), 7.02 (t, J = 8.6 Hz, 2H, CH_{ar}), 7.31 (dd, J = 5.6, 8.2 Hz, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.8, 20.9, 21.1 (3 OAc), 23.2 (NHAc), 49.3 (C-3), 51.0 (C-5), 52.8 (C-9), 53.5 (OMe), 66.1 (*C*H_2Ar), 68.0 (C-7), 68.9 (C-4), 69.7 (C-8), 72.8 (C-6), 98.5 (C-2), 115.2 (J = 21.7 Hz), 129.6 (J = 8.4 Hz), 132.9 (J = 2.9 Hz), 162.4 (J = 246.0 Hz) (6 C−Ar), 168.4, 170.2, 170.3, 170.4, 171.0 (6C, 6 CO). ESI-MS calcd for $C_{25}H_{31}FN_4O_{11}$ $[M + Na]^+$ 605.20; found m/z 605.22.

Methyl (Pentafluorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (11c). Compound **10** (60.0 mg, 0.12 mmol) was reacted with pentafluorobenzyl alcohol (60.0 mg, 0.30 mmol), *N*-iodosuccinimide (32.0 mg, 0.14 mmol), and triflic acid (4.00 μL, 7.00 mg, 0.04 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient *i*PrOH in petrol ether/DCM 8:4) to yield **11c** (47.0 mg, 66%) as a colorless oil. $[\alpha]^{20}_D$ −0.03 (*c* 0.5, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.90 (s, 3H, NHAc), 1.94 (dd, J = 10.4, 12.6 Hz, 1H, H-3a), 2.03, 2.19, 2.21 (3 s, 9H, 3 OAc), 2.59 (dd, J = 4.6, 12.8 Hz, 1H, H-3b), 3.30 (dd, J = 5.6, 13.5 Hz, 1H, H-9a), 3.59 (dd, J = 2.9, 13.5 Hz, 1H, H-9b), 3.86 (s, 3H, OMe), 4.12 (m, 2H, H-5, H-6), 4.43 (A of AB, J = 11.0 Hz, 1H, CH_2Ar), 4.87 (m, 1H, H-4), 4.90 (B of AB, J = 10.7 Hz, 1H, CH_2Ar), 5.19 (d, J = 9.3 Hz, 1H, NH), 5.39 (m, 2H, H-7, H-8). ^{13}C NMR ($CDCl_3$) δ 20.8, 20.9, 21.1 (3 OAc), 23.2 (NHAc), 37.7 (C-3), 49.4 (C-5), 51.0 (C-9), 53.0 (OMe), 54.0 (*C*H_2Ar), 67.8, 68.8, 69.3 (C-4, C-7, C-8), 72.9 (C-6), 98.7 (C-2), 167.8, 170.3 (5C, 5 CO). ESI-MS calcd for $C_{25}H_{27}F_5N_4O_{11}$ $[M + Na]^+$ 677.16; found m/z 677.32.

Methyl (2-Naphthyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (11d). Compound **10** (50.0 mg, 0.10 mmol) was reacted with 2-naphthalenemethanol (24.0 mg, 0.15 mmol), *N*-iodosuccinimide (27.0 mg, 0.12 mmol), and triflic acid (4.00 μL, 7.00 mg, 0.04 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient *i*PrOH in petrol ether/DCM 8:4) to yield **11d** (37.0 mg, 61%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.85 (s, 3H, NHAc), 2.09 (m, 1H, H-3a), 2.03, 2.15, 2.21 (3 s, 9H, 3 OAc), 2.26 (dd, J = 6.0, 13.5 Hz, 1H, H-9a), 3.59 (dd, J = 2.9, 13.5 Hz, 1H, H-9b), 3.67 (s, 3H, OMe), 4.14 (m, 2H, H-5, H-6), 4.63 (A of AB, J = 12.2 Hz, 1H, CH_2Ar), 4.89 (m, 1H, H-4), 4.96 (B of AB, J = 12.2 Hz, 1H, CH_2Ar), 5.34 (m, 2H, H-7, NH), 5.38 (m, 1H, H-8), 7.45−7.48 (m, 3H, CH_{ar}), 7.79−7.83 (m, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.3, 21.5 (3C, 3 OAc), 23.6 (NHAc), 38.5 (C-3), 49.9 (C-5), 51.3 (C-9), 53.1 (OMe), 67.4 (*C*H_2Ar), 68.4 (C-7), 69.4 (C-4), 70.2 (C-8), 73.3 (C-6), 126.1, 126.4, 126.5, 126.9, 128.1, 128.3, 128.4 (10 C−Ar), 170.7 (6C, 6 CO). ESI-MS calcd for $C_{29}H_{34}N_4O_{11}$ $[M + Na]^+$ 637.33; found m/z 637.20.

Methyl (2,3-Dichlorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (11e). Compound **10** (45.0 mg, 0.09 mmol) was reacted with 2,3-dichlorobenzyl alcohol (24.0 mg, 0.13 mmol), *N*-iodosuccinimide (24.0 mg, 0.12 mmol), and triflic acid (3.00 μL, 6.00 mg, 0.04 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient *i*PrOH in petrol ether/DCM 8:4) to yield **11e** (30.0 mg, 54%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.83 (s, 3H, NHAc), 2.01 (t, J = 12.9 Hz, 1H, H-3a), 1.97, 2.08, 2.11 (3 s, 9H, 3 OAc), 2.63 (dd, J = 4.7, 12.9 Hz, 1H, H-3b), 3.19 (dd, J = 6.0, 13.6 Hz, 1H, H-9a), 3.50 (dd, J = 3.0, 13.6 Hz, 1H, H-9b), 3.72 (s, 3H, OMe), 4.05 (m, 2H, H-5, H-6), 4.50, 4.82 (A, B of AB, J = 13.0 Hz, 2H, CH_2Ar), 4.85 (m, 1H, H-4), 5.13 (d, J = 8.0 Hz, 1H, N*H*Ac), 5.24 (m, 2H, H-7, H-8), 7.14 (t, J = 8.0 Hz, 1H, CH_{ar}), 7.34 (d, J = 8.1 Hz, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.3, 21.5 (3C, 3 OAc), 23.6 (NHAc), 38.2 (C-3), 49.9 (C-5), 51.2 (C-9), 53.4 (OMe), 64.7 (*C*H_2Ar), 68.4 (C-8), 69.3 (C-4), 70.5 (C-7), 73.5 (C-6), 109.6 (C-2), 127.6, 127.7, 130.0 (6C, 6 C−Ar), 168.5, 170.5, 170.6, 170.7, 171.4 (5 CO). ESI-MS calcd for $C_{25}H_{30}Cl_2N_4O_{11}$ $[M + Na]^+$ 655.13; found m/z 655.07.

Methyl (2,3-Difluorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (11f). Compound **10** (86.0 mg, 0.17 mmol) was reacted with 2,3-difluorobenzyl alcohol (29.0 μL, 37.0 mg, 0.26 mmol), *N*-iodosuccinimide (46.0 mg, 0.21 mmol), and triflic acid (6.00 μL, 10.0 mg, 0.07 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient *i*PrOH in petrol ether/DCM 8:4) to yield **11f** (67.0 mg, 66%) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.82 (s, 3H, NHAc), 1.95 (m, 1H, H-3a), 1.96, 2.10, 2.13 (3 s, 9H, 3 OAc), 2.58 (m, 1H, H-3b), 3.20 (m, 1H, H-9a), 3.50 (m, 1H, H-9b), 3.72 (s, 3H, OMe), 4.06 (m, 2H, H-5, H-6), 4.46, 4.75 (A, B of AB, J = 12.0 Hz, 2H, CH_2Ar), 4.82 (m, 1H, H-4), 5.29 (m, 2H, H-8, H-7), 7.05 (m, 3H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.3, 21.6 (3C, 3 OAc), 23.6 (NHAc), 38.2 (C-3), 49.8 (C-5), 51.3 (C-9), 53.3 (OMe), 60.8 (*C*H_2Ar), 68.4 (C-7), 69.3 (C-8), 70.3 (C-4), 73.4 (C-6), 99.1 (C-2), 117.4, 119.1 (J = 17.0 Hz), 124.3, 125.5 (6 C−Ar), 168.5, 170.5, 170.6, 170.8, 171.4 (5 CO). ESI-MS calcd for $C_{25}H_{30}F_2N_4O_{11}$ $[M + 2Na]^+$ 645.99; found m/z 645.26.

Methyl (4-Chlorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (12a). Compound **11a** (55.0 mg, 0.09 mmol) was reacted with *p*-chlorobenzoyl chloride (46.0 μL, 63.0 mg, 0.36 mmol) and triphenylphosphine (47.0 mg, 0.18 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient of MeOH in DCM) to yield **12a** (35.0 mg, 59%) as a yellow solid. $[\alpha]^{20}_D$ −0.01 (*c* 2.9, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.89 (s, 3H, NHAc), 2.05 (m, 4H, H-3a, OAc), 2.14, 2.27 (2 s, 6H, 2 OAc), 2.67 (dd, J = 4.6, 12.8 Hz, 1H, H-3b), 2.92 (dt, J = 3.3, 14.9 Hz, 1H, H-9a), 3.65 (s, 3H, OMe), 4.05 (dd, J = 2.0, 10.7 Hz, 1H, H-6), 4.21 (q, J = 10.4 Hz, 1H, H-5), 4.36 (ddd, J = 3.0, 8.7, 15.1 Hz, 1H, H-9b), 4.41, 4.78 (A, B of AB, J = 12.3 Hz, 2H, CH_2Ar), 4.84 (m, 1H, H-4), 5.15 (dd, J = 2.0, 10.0 Hz, 1H, H-7), 5.31 (m, 2H, N*H*Ac, H-8), 7.10 (dd, J = 4.0, 8.5 Hz, 1H, NH), 7.29 (m, 4H, CH_{ar}), 7.41, 7.77 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.9, 21.2, 21.3 (3 OAc), 23.2 (NHAc), 38.1 (C-3), 38.4 (C-9), 49.5 (C-5), 52.7 (OMe), 66.1 (*C*H_2Ar), 67.9 (C-7), 68.2 (C-8), 69.0 (C-4), 72.2 (C-6), 98.4 (C-2), 128.4, 128.5, 128.8, 129.2, 132.0, 132.6, 133.5, 135.7, 137.7 (12 C−Ar), 168.1, 170.4, 171.2, 172.6 (6C, 6 CO). ESI-MS calcd for $C_{32}H_{36}Cl_2N_2O_{12}$ $[M + Na]^+$ 733.16; found m/z 733.25.

Methyl (4-Fluorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (12b). Compound **11b** (52.0 mg, 0.09 mmol) was reacted with *p*-chlorobenzoyl chloride (46.0 μL, 63.0 mg, 0.36 mmol) and triphenylphosphine (52.0 mg, 0.19 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient of MeOH in DCM) to yield **12b** (37.0 mg, 60%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.85 (s, 3H, NHAc), 2.00 (m, 1H, H-3a), 2.03, 2.11, 2.26 (3 s, 9H, 3 OAc), 2.68 (dd, J = 4.5, 12.7 Hz, 1H, H-3b), 2.96 (dt, J = 3.5, 15.0 Hz, 1H, H-9a), 3.61 (s, 3H, OMe), 4.12 (d, J = 10.7 Hz, 1H, H-6), 4.23 (q, J = 10.2 Hz, 1H, H-5), 4.36 (ddd, J = 3.0, 8.7, 15.3 Hz, 1H, H-9b), 4.40, 4.78 (A, B of AB, J = 11.9 Hz, 2H, CH_2Ar), 4.87 (m, 1H, H-4), 5.20 (dd, J = 1.1, 9.9 Hz, 1H, H-7), 5.35 (dt, J = 2.9, 9.9 Hz, 1H, H-8), 5.99 (m, 1H, N*H*Ac), 7.00 (t, J = 8.7 Hz, 2H, CH_{ar}), 7.17 (dd, J = 3.8 Hz, 8.1 Hz, 1H, NH), 7.49 (dd, J = 5.4 Hz, 8.5 Hz, 1H, CH_{ar}), 7.38, 7.76 (AA′, BB′ of AA′BB′, J = 8.4 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.2, 21.3 (3 OAc), 23.1 (NHAc), 38.3 (C-3), 38.5 (C-9), 49.4 (C-5), 52.6 (OMe), 66.1 (*C*H_2Ar), 68.0 (C-7), 68.2 (C-8), 69.2 (C-4), 72.2 (C-6), 98.4 (C-2), 115.0, 115.2 (J = 21.3 Hz), 128.6, 129.6, 129.7 (J = 7.5 Hz), 132.0, 132.8, 132.9, 137.7, 161.3, 163.5 (J = 275.1 Hz), 168.1, 166.3 (J = 237.5 Hz) (12 C−Ar), 170.39, 170.4, 171.0, 172.5 (6C, 6 CO). ESI-MS calcd for $C_{32}H_{36}ClFN_2O_{12}$ $[M + Na]^+$ 717.19; found m/z 717.34.

Methyl (Pentafluorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (12c). Compound **11c** (47.0 mg, 0.07 mmol) was reacted with *p*-chlorobenzoyl chloride (36.0 μL, 49.0 mg, 0.28 mmol) and triphenylphosphine (41.0 mg, 0.16 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient of MeOH in DCM) to yield **12c** (25.0 mg, 45%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.86 (s, 3H, NHAc), 1.94 (m, 1H, H-3a), 2.02, 2.16, 2.19 (3 s, 9H, 3 OAc), 2.60 (dd, *J* = 4.5, 12.8 Hz, 1H, H-3b), 3.55 (dd, *J* = 6.3, 12.3 Hz, 1H, H-9a), 3.81 (m, 4H, OMe, H-9b), 4.07 (d, *J* = 10.3 Hz, 1H, H-6), 4.20 (dd, *J* = 2.0, 10.8 Hz, 1H, H-5), 4.45, 4.93 (A, B of AB, *J* = 11.3 Hz, 2H, CH_2Ar), 5.37 (dd, *J* = 2.1, 8.1 Hz, 1H, H-7), 5.46 (m, 1H, H-8), 5.68 (d, *J* = 9.6 Hz, 1H, N*H*Ac), 7.38, 8.00 (AA′, BB′ of AA′BB′, *J* = 8.4 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.0, 21.2, 21.3 (3 OAc), 23.3 (NHAc), 37.8 (C-3), 43.7 (C-9), 49.5 (C-5), 53.1 (OMe), 54.1 (*C*H_2Ar), 68.3, 69.0 (C-4, C-7), 70.0 (C-8), 72.9 (C-6), 98.8 (C-2), 128.8, 128.9, 129.1, 131.5, 132.9, 133.2, 133.3 (12 C−Ar), 167.6, 167.9, 168.7, 170.3, 170.4, 170.6 (6 CO). ESI-MS calcd for $C_{32}H_{32}ClF_5N_2O_{12}$ $[M + Cl]^-$ 801.45; found *m*/*z* 801.36.

Methyl (2-Naphthyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (12d). Compound **11d** (50.0 mg, 0.08 mmol) was reacted with *p*-chlorobenzoyl chloride (42.0 μL, 57.0 mg, 0.32 mmol) and triphenylphosphine (47.0 mg, 0.18 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient of MeOH in DCM) to yield **12d** (25.0 mg, 47%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.87 (s, 3H, NHAc), 2.04 (s, 3H, OAc), 2.10 (t, *J* = 12.4 Hz, 1H, H-3a), 2.14, 2.24 (2 s, 6H, 2 OAc), 2.72 (dd, *J* = 4.5, 12.8 Hz, 1H, H-3b), 2.92 (m, 1H, H-9a), 3.59 (s, 3H, OMe), 4.11 (m, 1H, H-6), 4.23 (q, *J* = 10.3 Hz, 1H, H-5), 4.34 (ddd, *J* = 3.0, 8.5, 15.2 Hz, 1H, H-9b), 4.63 (A of AB, *J* = 12.3 Hz, 1H, CH_2Ar), 4.87 (dt, *J* = 4.6, 12.3 Hz, 1H, H-4), 4.98 (B of AB, *J* = 12.3 Hz, 1H, CH_2Ar), 5.17 (d, *J* = 9.8 Hz, 1H, H-7), 5.34 (d, *J* = 9.9 Hz, 1H, H-8), 5.50 (m, 1H, N*H*Ac), 7.11 (m, 1H, NH), 7.39 (AA′ of AA′BB′, *J* = 8.5 Hz, 2H, CH_{ar}), 7.44−7.47 (m, 3H, CH_{ar}), 7.76 (BB′ of AA′BB′, *J* = 8.5 Hz, 2H, CH_{ar}), 7.75−7.83 (m, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.3, 21.5, 21.6 (3 OAc), 23.5 (NHAc), 38.6 (C-3), 38.9 (C-9), 49.9 (C-5), 53.0 (OMe), 67.4 (*C*H_2Ar), 68.4 (C-7), 68.7 (C-8), 69.5 (C-4), 72.6 (C-6), 98.9 (C-2), 126.2, 126.3, 126.5, 127.0, 128.0, 128.3, 128.9, 129.0, 129.2, 132.4, 133.1, 133.3, 133.5, 135.1 (14 C−Ar), 166.6, 168.7, 170.8, 171.5, 172.9 (6C, 6 CO). ESI-MS calcd for $C_{36}H_{39}ClN_2O_{12}$ $[M + Na]^+$ 749.22; found *m*/*z* 749.25.

Methyl (2,3-Dichlorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (12e). Compound **11e** (47.0 mg, 0.07 mmol) was reacted with *p*-chlorobenzoyl chloride (38.0 μL, 52.0 mg, 0.30 mmol) and triphenylphosphine (43.0 mg, 0.16 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient of MeOH in DCM) to yield **12e** (25.0 mg, 47%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.78 (t, *J* = 12.4 Hz, 1H, H-3a), 1.80 (s, 3H, NHAc), 1.97, 2.04, 2.17 (3 s, 9H, 3 OAc), 2.63 (dd, *J* = 4.8, 12.8 Hz, 1H, H-3b), 2.90 (ddd, *J* = 3.3, 3.7, 15.1 Hz, 1H, H-9a), 3.64 (s, 3H, OMe), 4.01 (d, *J* = 10.8 Hz, 1H, H-6), 4.15 (q, *J* = 10.4 Hz, 1H, H-5), 4.23 (m, 1H, H-9b), 4.49 (A of AB, *J* = 13.0 Hz, 1H, *C*H_2Ar), 4.30 (m, 2H, H-4, *C*H_2Ar), 5.11 (d, *J* = 1.6 Hz, 1H, H-7), 5.20 (m, 1H, H-8), 5.55 (d, *J* = 8.8 Hz, 1H, N*H*Ac), 7.03 (dd, *J* = 3.8 Hz, 1H, NH), 7.13 (t, *J* = 7.8 Hz, 1H, CH_{ar}), 7.39 (m, 2H, CH_{ar}), 7.68, 7.93 (AA′, BB′ of AA′BB′, *J* = 8.5 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.3, 21.5, 21.6 (3 OAc), 23.5 (NHAc), 38.2 (C-3), 39.0 (C-9), 49.9 (C-5), 53.2 (OMe), 64.8 (*C*H_2Ar), 68.4 (C-8), 68.8 (C-7), 69.5 (C-4), 72.7 (C-6), 110.0 (C-2), 127.6, 128.1, 130.0, 131.8, 133.0, 136.0, 138.1 (12 C−Ar), 160.0, 168.0, 170.6, 170.9, 171.5, 172.8 (6 CO). ESI-MS calcd for $C_{32}H_{35}Cl_3N_2O_{12}$ $[M + Na]^+$ 767.13; found *m*/*z*: 767.12.

Methyl (2,3-Difluorobenzyl 5-Acetamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (12f). Compound **11f** (67.0 mg, 0.11 mmol) was reacted with *p*-chlorobenzoyl chloride (57.0 μL, 78.0 mg, 0.45 mmol) and triphenylphosphine (65.0 mg, 0.25 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient of MeOH in DCM) to yield **12f** (44.0 mg, 55%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.80 (s, 3H, NHAc), 2.04 (m, 4H, H-3a, OAc), 2.14, 2.27 (2 s, 6H, 2 OAc), 2.65 (dd, *J* = 4.6, 12.8 Hz, 1H, H-3b), 2.97 (dt, *J* = 3.5, 15.0 Hz, 1H, H-9a), 3.74 (s, 3H, OMe), 4.08 (m, 1H, H-6), 4.23 (q, *J* = 10.4 Hz, 1H, H-5), 4.33 (m, 1H, H-9b), 4.52, 4.84 (A, B of AB, *J* = 12.0 Hz, 2H, CH_2Ar), 5.17 (dd, *J* = 1.9, 9.8 Hz, 1H, H-7), 5.31 (m, 1H, H-8), 5.43 (m, 1H, N*H*Ac), 7.75 (m, 4H, NH, CH_{ar}), 7.39, 7.77 (AA′, BB′ of AA′BB′, *J* = 8.5 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.3, 21.5, 21.6 (3 OAc), 23.5 (NHAc), 38.3 (C-3), 38.9 (C-9), 49.9 (C-5), 53.2 (OMe), 60.8 (*C*H_2Ar), 68.3 (C-8), 68.7 (C-7), 69.4 (C-4), 72.7 (C-6), 98.0 (C-2), 117.3, 117.4 (*J* = 17.0 Hz), 125.7, 128.8, 129.2, 132.4, 132.5 (*J* = 9.0 Hz), 133.0, 138.1 (12 C−Ar), 166.7, 168.4, 170.7, 170.9, 171.6, 172.9 (6 CO). ESI-MS calcd for $C_{32}H_{35}ClF_2N_2O_{12}$ $[M + Na]^+$ 735.18; found *m*/*z* 735.15.

Sodium (4-Chlorobenzyl 5-Acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (13a). Compound **12a** (25.0 mg, 0.03 mmol) was treated with 10% aqueous NaOH (0.2 mL) in MeOH (1.0 mL). The crude product was purified by chromatography on RP-18 (5% gradient of MeOH in H_2O) to yield **13a** as a white solid (15.0 mg, 50%). $[\alpha]^{20}_D$ −0.25 (*c* 0.41, H_2O). ^{1}H NMR (500 MHz, CD_3OD) δ 1.55 (dd, *J* = 3.3, 11.9 Hz, 1H, H-3a), 1.90 (s, 3H, NHAc), 2.81 (dd, *J* = 2.0, 12.2 Hz, 1H, H-3b), 3.34 (dd, *J* = 1.8, 9.0 Hz, 1H, H-7), 3.41 (dd, *J* = 7.8, 13.6 Hz, 1H, H-9a), 3.55−3.63 (m, 3H, H-4, H-5, H-6), 3.67 (dd, *J* = 3.1 Hz, 13.6 Hz, 1H, H-9b), 3.94 (m, 1H, H-8), 4.40, 4.70 (A, B of AB, *J* = 11.6 Hz, 2H, CH_2Ar), 7.18, 7.25 (AA′, BB′ of AA′BB′, *J* = 8.4 Hz, 4H, CH_{ar}), 7.36, 7.72 (AA′, BB′ of AA′BB′, *J* = 8.6 Hz, 4H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 22.6 (NHAc), 42.7 (C-3), 44.6 (C-9), 54.2 (C-5), 66.5 (*C*H_2Ar), 69.6 (C-4), 71.2 (C-8), 72.5 (C-7), 74.4 (C-6), 102.1 (C-2), 129.2, 129.7, 130.1, 130.6, 134.0, 134.5, 138.6, 138.8 (12 C−Ar), 169.2, 174.3, 175.5 (3 CO). HRMS calcd for $C_{25}H_{28}Cl_2N_2O_9$ $[M - H]^-$ 615.0891; found 615.0888.

Sodium (4-Fluorobenzyl 5-Acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (13b). Compound **12b** (37.0 mg, 0.05 mmol) was treated with 10% aqueous NaOH (0.3 mL) in MeOH (1.2 mL). The crude product was purified by chromatography on RP-18 (5% gradient of MeOH in H_2O) to yield **13b** as a white solid (21.1 mg, 70%). $[\alpha]^{20}_D$ −0.22 (*c* 0.33, H_2O). ^{1}H NMR (500 MHz, CD_3OD) δ 1.54 (m, 1H, H-3a), 1.90 (s, 3H, NHAc), 2.80 (m, 1H, H-3b), 3.35 (m, 1H, H-7), 3.42 (m, 1H, H-9a), 3.58−3.69 (m, 4H, H-4, H-5, H-6, H-9b), 3.96 (m, 1H, H-8), 4.39, 4.69 (A, B of AB, *J* = 11.2 Hz, 2H, CH_2Ar), 6.90 (t, *J* = 8.8 Hz, 2H, CH_{ar}), 7.27 (m, 2H, CH_{ar}), 7.35 (m, 2H, CH_{ar}), 7.72 (BB′ of AA′BB′, *J* = 8.5 Hz, 2H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 22.7 (NHAc), 42.7 (C-3), 44.5 (C-9), 54.2 (C-5), 66.7 (*C*H_2Ar), 69.6 (C-4), 71.4 (C-8), 72.5 (C-7), 74.4 (C-6), 102.1 (C-2), 115.8 (*J* = 12.5 Hz), 130.1, 129.7, 131.1 (*J* = 21.3 Hz), 138.6, 162.0 (12 C−Ar), 164.6, 169.3, 174.4 (3 CO). HRMS calcd for $C_{25}H_{28}ClFN_2O_9$ $[M - H]^-$ 577.1367; found 577.1369.

Sodium (Pentafluorobenzyl 5-Acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (13c). Compound **12c** (25.0 mg, 0.03 mmol) was treated with 10% aqueous NaOH (0.2 mL) in MeOH (1.0 mL). The crude product was purified by chromatography on RP-18 (5% gradient of MeOH in H_2O) to yield **13c** as a white solid (8.00 mg, 39%). $[\alpha]^{20}_D$ −0.02 (*c* 0.32, H_2O). ^{1}H NMR (500 MHz, D_2O) δ 1.63 (t, *J* = 12.1 Hz, 1H, H-3a), 1.97 (s, 3H, NHAc), 2.71 (dd, *J* = 4.7, 12.4 Hz, 1H, H-3b), 3.50 (m, 2H, H-7, H-9a), 3.65 (ddd, *J* = 4.7, 9.5, 11.9 Hz, 1H, H-4), 3.74−3.79 (m, 3H, H-5, H-6, H-9b), 3.88 (ddd, *J* = 2.9, 7.8, 8.8 Hz, 1H, H-8), 4.66, 4.86 (A, B of AB, *J* = 11.7 Hz, 2H, CH_2Ar), 7.50, 7.73 (AA′, BB′ of AA′BB′, *J* = 8.6 Hz, 4H, CH_{ar}). ^{13}C NMR (D_2O) δ 21.9 (NHAc), 40.3 (C-3), 42.6 (C-9), 51.8 (C-5), 54.1 (*C*H_2Ar), 68.1 (C-4), 69.8 (C-7), 70.3 (C-8), 72.8 (C-6), 101.0 (C-2), 128.7, 128.7, 132.2, 136.2, 137.5, 139.1

(12 C−Ar), 170.1, 172.8, 175.0 (3 CO). HRMS calcd for $C_{25}H_{23}ClF_5N_2NaO_9$ $[M - H]^-$ 625.1018; found 625.1015.

Sodium (2-Naphthyl 5-Acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (13d). Compound **12d** (25.0 mg, 0.03 mmol) was treated with 10% aqueous NaOH (0.2 mL) in MeOH (1.0 mL). The crude product was purified by chromatography on RP-18 (5% gradient of MeOH in H_2O) to yield **13d** as a white solid (14.0 mg, 70%). $[\alpha]^{20}_D$ −0.37 (*c* 0.46, H_2O). ^{1}H NMR (500 MHz, CD_3OD) δ 1.60 (m, 1H, H-3a), 1.90 (s, 3H, NHAc), 2.84 (d, J = 11.8 Hz, H-3b), 3.35 (d, J = 8.9 Hz, H-7), 3.42 (dd, J = 7.7, 13.7 Hz, H-9a), 3.60−3.68 (m, 4H, H-4, H-5, H-6, H-9b), 4.60 (A of AB, J = 11.5 Hz, 1H, CH_2Ar), 3.96 (m, 1H, H-8), 4.89 (B of AB, J = 11.5 Hz, 1H, CH_2Ar), 7.31−7.36 (m, 4H, CH_{ar}), 7.40 (dd, J = 1.5, 8.4 Hz, 1H, CH_{ar}), 7.67−7.72 (m, 6H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 21.7 (NHAc), 43.3 (C-3), 43.4 (C-9), 53.1 (C-5), 68.6 (*C*H_2Ar), 71.3 (C-7), 72.0 (C-4), 73.3 (C-6), 110.0 (C-2), 125.7, 125.9, 126.3, 126.4, 127.6, 127.7, 127.9, 128.6, 129.1 (16 C−Ar), 174.6, 175.3 (3C, CO). HRMS calcd for $C_{29}H_{30}ClN_2NaO_9$ $[M + Na]^+$ 631.1438; found 631.1435.

Sodium (2,3-Dichlorobenzyl 5-Acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (13e). Compound **12e** (25.0 mg, 0.03 mmol) was treated with 10% aqueous NaOH (0.2 mL) in MeOH (1.0 mL). The crude product was purified by chromatography on RP-18 (5% gradient of MeOH in H_2O) to yield **13e** as a white solid (10.0 mg, 50%). $[\alpha]^{20}_D$ −0.19 (*c* 0.53, H_2O). ^{1}H NMR (500 MHz, CD_3OD) δ 1.69 (t, J = 11.8 Hz, 1H, H-3a), 1.93 (s, 3H, NHAc), 2.01 (dd, J = 9.1, 11.8 Hz, 1H, H-3b), 3.36 (dd, J = 1.7, 9.0 Hz, 1H, H-7), 3.45 (dd, J = 7.6, 13.6 Hz, 1H, H-9a), 3.59 (m, 1H, H-6), 3.65−3.70 (m, 3H, H-4, H-5, H-9b), 3.92 (m, 1H, H-8), 4.65, 4.89 (A, B of AB, J = 13.6 Hz, 2H, CH_2Ar), 7.18 (t, J = 7.9 Hz, 1H, CH_{ar}), 7.32 (dd, J = 1.5, 8.0 Hz, 1H, CH_{ar}), 7.39 (AA′ of AA′BB′, J = 8.5 Hz, 2H, CH_{ar}), 7.49 (dd, J = 1.1, 7.7 Hz, 1H, CH_{ar}), 7.74 (BB′ of AA′BB′, J = 8.5 Hz, 2H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 21.6 (NHAc), 41.5 (C-3), 43.4 (C-9), 53.1 (C-5), 63.5 (*C*H_2Ar), 68.6 (C-4), 70.2 (C-8), 71.5 (C-7), 73.4 (C-6), 102.5 (C-2), 113.3, 117.0, 118.4, 127.4, 127.5, 128.6, 128.9, 129.0, 139.6 (12 C−Ar), 163.8, 174.5 (3C, 3 CO). HRMS calcd for $C_{25}H_{27}Cl_3N_2O_9$ $[M + Na]^+$ 627.0682; found 627.0683.

Sodium (2,3-Difluorobenzyl 5-Acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (13f). Compound **12f** (44.0 mg, 0.06 mmol) was treated with 10% aqueous NaOH (0.3 mL) in MeOH (1.5 mL). The crude product was purified by chromatography on RP-18 (5% gradient of MeOH in H_2O) to yield **13f** as a white solid (23.0 mg, 64%). $[\alpha]^{20}_D$ −0.18 (*c* 1.13, H_2O). ^{1}H NMR (500 MHz, CD_3OD) δ 1.56 (m, 1H, H-3a), 1.90 (s, 3H, NHAc), 2.84 (dd, J = 3.0, 12.2 Hz, 1H, H-3b), 3.37 (d, J = 9.0 Hz, 1H, H-7), 3.46 (dd, J = 7.7, 13.6 Hz, 1H, H-9a), 3.59 (m, 1H, H-6), 3.63−3.70 (m, 3H, H-4, H-5, H-9b), 3.96 (m, 1H, H-8), 4.60, 4.85 (A, B of AB, J = 12.2 Hz, 2H, CH_2Ar), 7.04 (m, 2H, CH_{ar}), 7.24 (t, J = 6.5 Hz, 1H, CH_{ar}), 7.38, 7.75 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 21.6 (NHAc), 41.5 (C-3), 43.5 (C-9), 53.1 (C-5), 59.2 (*C*H_2Ar), 68.6 (C-4), 70.3 (C-8), 71.4 (C-7), 73.4 (C-6), 101.0 (C-2), 115.9, 116.1 (J = 17.3 Hz), 124.2, 125.3, 128.6, 129.1, 133.5, 137.6 (12 C−Ar), 168.3, 173.0, 174.5 (3 CO). HRMS calcd for $C_{25}H_{27}ClF_2N_2O_9$ $[M + Na]^+$ 595.1273; found 595.1272.

Methyl (Methyl 5-*tert*-Butyloxycarbonylamino-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (14). Compound **10** (85.0 mg, 0.17 mmol) was dissolved in dry THF (0.7 mL) under argon. Boc_2O (74.0 mg, 0.34 mmol) was added to the reaction mixture, followed by DMAP (4.50 mg, 0.02 mmol). The reaction mixture was heated up to 60 °C for 5 h. After cooling to rt, MeOH (0.7 mL) and $N_2H_4 \cdot H_2O$ (52 μL, 1.1 mmol) were added and stirring was continued for 16 h. The reaction mixture was washed successively with 0.1 M HCl (1 × 5 mL), 0.5 M $CuSO_4$ (1 × 5 mL), saturated aqueous $NaHCO_3$ (2 × 5 mL), and H_2O (1 × 5 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give a yellow oil. The crude product was reacted with acetic anhydride (0.9 mL) in dry pyridine (1.7 mL). A catalytic amount of DMAP was added, and stirring was continued at rt. After 18 h, the reaction mixture was washed with $CuSO_4$ (0.5 M, 4 × 5 mL), saturated aqueous $NaHCO_3$ (1 × 5 mL), and H_2O (1 × 5 mL). The organic layer was dried over Na_2SO_4, filtered, and the solvent was evaporated. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **14** (72 mg, 76%) as a white foam. $[\alpha]^{20}_D$ 0.31 (*c* 1.68, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.37 (s, 9H, *t*-Butyl), 1.95 (m, 1H, H-3a), 2.02 (s, 3H, SMe), 2.10, 2.14, 2.17 (3 s, 9H, 3 OAc), 2.73 (dd, J = 4.6, 12.7 Hz, 1H, H-3b), 3.25 (dd, J = 6.0, 13.5 Hz, 1H, H-9a), 3.61 (dd, J = 3.2, 13.5 Hz, 1H, H-9b), 3.73 (m, 1H, H-6), 3.80 (m, 4H, OMe, H-5), 4.25 (d, J = 10.4 Hz, 1H, NH), 4.78 (m, 1H, H-4), 5.29 (m, 1H, H-8), 5.41 (m, 1H, H-7). ^{13}C NMR ($CDCl_3$) δ 12.1 (SMe), 20.8, 21.0, 21.3 (3 OAc), 27.9 (3C, C-(*C*H_3)$_3$), 38.0 (C-3), 50.4 (C-9), 50.6 (C-5), 52.9 (OMe), 67.6 (C-7), 68.1 (C-8), 70.0 (C-4), 73.7 (C-6), 80.2 (C-2), 83.0 (*C*(CH$_3$)$_3$), 155.2 (CONH), 168.2, 170.0, 170.6, 174.0 (4 CO). ESI-MS calcd for $C_{22}H_{34}N_4O_{11}S$ $[M + Na]^+$ 585.19; found m/z 585.15.

Methyl (Methyl 5-Amino-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (15). Compound **14** (120 mg, 0.21 mmol) was dissolved in 4 M PhOH (in abs DCM; 7.5 mL) and 4 M TMSCl (in abs DCM; 1.5 mL). The reaction mixture was stirred at rt for 2 h. The reaction mixture was washed with saturated aqueous $NaHCO_3$ (2 × 5 mL) and H_2O (1 × 5 mL). The organic layer was dried over Na_2SO_4, filtered, and the solvent was evaporated. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 8:1) to yield **15** as a white foam (74 mg, 70%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.01 (s, 3H, SMe), 2.04 (m, 1H, H-3a), 2.10, 2.11, 2.19 (3 s, 9H, 3 OAc), 2.71 (dd, J = 4.7, 12.8 Hz, 1H, H-3b), 3.38 (d, J = 10.5 Hz, 1H, H-6), 3.66 (dd, J = 3.3, 13.3 Hz, 1H, H-9a), 3.70 (dd, J = 2.8, 13.3 Hz, 1H, H-9b), 3.76 (dd, J = 1.3, 9.4 Hz, 1H, H-7), 3.78 (s, 3H, OMe), 3.98 (dt, J = 8.0, 10.5 Hz, 1H, H-5), 4.93 (dd, J = 6.9, 10.7 Hz, 1H, H-4), 5.26 (t, J = 3.9, 9.4 Hz, 1H, H-8), 5.94 (d, J = 7.8 Hz, 2H, NH_2). ^{13}C NMR ($CDCl_3$) δ 12.0 (SMe), 21.1, 21.2, 23.2 (3 OAc), 37.7 (C-3), 51.2, 52.1, 52.9 (3C, C-5 C-9, OMe), 66.8, 69.3, 70.0 (3C, C-4, C-7, C-8), 75.3 (C-6), 82.2 (C-2), 167.9, 170.2, 172.4, 172.9 (4 CO). ESI-MS calcd for $C_{17}H_{26}N_4O_9S$ $[M + Na]^+$ 485.14; found m/z 485.13.

Methyl (Methyl 5-Fluoroacetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (16a). Compound **15** (74.0 mg, 0.16 mmol) was dissolved in dry DCM (1.8 mL) and cooled to 0 °C. Then, monofluoroacetic chloride was added dropwise (30.0 mg, 31.0 μL, 0.32 mmol), followed by the addition of NEt_3 (324 mg, 0.45 mL, 3.2 mmol) and DMAP (9.8 mg, 0.08 mmol). Stirring was continued overnight, and the reaction mixture was allowed to come to rt. The brown solution was washed with saturated aqueous $NaHCO_3$ (3 × 5 mL), saturated aqueous NaCl (1 × 5 mL), and H_2O (1 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **16a** (71 mg, 85%). $[\alpha]^{20}_D$ 0.34 (*c* 1.2, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.98 (m, 1H, H-3a), 2.03 (s, 3H, SMe), 2.12, 2.15, 2.21 (3 s, 9H, 3 OAc), 2.79 (dd, J = 4.7, 12.8 Hz, 1H, H-3b), 3.24 (dd, J = 4.8, 13.6 Hz, 1H, H-9a), 3.64 (dd, J = 2.8, 13.6 Hz, 1H, H-9b), 3.84 (s, 3H, OMe), 3.90 (dd, J = 1.9, 10.7 Hz, 1H, H-6), 4.14 (m, 1H, H-5), 4.71 (m, 2H, CH_2F), 4.92 (td, J = 4.7, 11.6 Hz, 1H, H-4), 5.32 (m, 2H, H-7, H-8), 6.09 (dd, J = 3.3, 10.3 Hz, 1H, NH). ^{13}C NMR ($CDCl_3$) δ 12.2 (SMe), 20.9 (3C, OAc), 37.8 (C-3), 48.6 (C-5), 50.6 (C-9), 53.1 (OMe), 67.8 (C-7), 69.4, 69.6 (2C, C-4, C-8), 74.1 (C-6), 80.8 (J = 186.1 Hz, *C*H_2F), 83.1 (C-2), 168.2, 170.2, 170.6 (5C, CO). ESI-MS calcd for $C_{19}H_{27}FN_4O_{10}S$ $[M + Na]^+$ 545.14; found m/z 545.15.

Methyl (Methyl 5-Chloroacetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (16b). Compound **15** (55.0 mg, 0.12 mmol) was dissolved in dioxane/water (0.5 mL/0.1 mL), treated with triethylamine

(48.6 mg, 34 μL, 0.48 mmol), and cooled to 0 °C. Then, chloroacetic anhydride (41.0 mg, 0.48 mmol) was added, and stirring was continued at rt for 3 h. The reaction mixture was diluted with $CHCl_3$ (10.0 mL) and washed successively with saturated aqueous $NaHCO_3$ (3 × 5 mL) and H_2O (1 × 5 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (gradient, PE:EA; 1:1 to 1:2) to yield **16b** as a white foam (42 mg, 66%). $[\alpha]^{20}_D$ 0.33 (*c* 0.95, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.91 (t, *J* = 12.2 Hz, 1H, H-3a), 1.96 (s, 3H, SMe), 2.06, 2.08, 2.13 (3 s, 9H, 3 OAc), 2.72 (dd, *J* = 4.7 Hz, 12.8 Hz, 1H, H-3b), 3.18 (m, 1H, H-9a), 3.59 (m, 1H, H-9b), 3.78 (s, 3H, OMe), 3.85 (A of AB, *J* = 15.0 Hz, 1H, C*H*$_2$Cl), 3.86 (m, 1H, H-6), 3.93 (B of AB, *J* = 15.0 Hz, 1H, C*H*$_2$Cl), 4.03 (m, 1H, H-5), 4.90 (dt, *J* = 4.7 Hz, 11.5 Hz, 1H, H-4), 5.24 (m, 2H, H-7, H-8), 6.28 (d, *J* = 10.1 Hz, 1H, NH). ^{13}C NMR ($CDCl_3$) δ 12.1 (SMe), 20.8, 20.9, 21.1 (3 OAc), 37.9 (C-3), 42.5 (*C*H$_2$Cl), 49.7 (C-9), 50.6 (C-5), 53.1 (OMe), 67.1, 67.9, 69.2 (3C, C-4, C-7, C-8), 74.2 (C-6), 83.1 (C-2), 166.6, 167.8, 170.3, 170.6 (5C, CO). ESI-MS calcd for $C_{19}H_{27}ClN_4O_{10}S$ $[M + Na]^+$ 561.10; found *m*/*z* 561.37.

Methyl (Methyl 5-*o*-Nitrotoluenesulfonamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (16c). Compound **15** (73.0 mg, 0.16 mmol) was dissolved in dry DCM (3.0 mL), and it was cooled to 0 °C. Nosylchloride (105 mg, 0.47 mmol), NEt_3 (34.0 μL, 48.0 mg, 0.47 mmol), and DMAP (10.0 mg, 0.08 mmol) were added successively. The reaction mixture was stirred at rt overnight. Then it was washed with saturated aqueous $NaHCO_3$ (2 × 5 mL) and H_2O (1 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **16c** (81 mg, 78%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.85 (m, 1H, H-3a), 2.02 (s, 3H, SMe), 2.10, 2.13, 2.21 (3 s, 9H, 3 OAc), 2.80 (m, 1H, H-3b), 3.32 (dd, *J* = 6.2, 13.4 Hz, 1H, H-9a), 3.57 (dd, *J* = 3.2, 13.4 Hz, 1H, H-9b), 3.80 (m, 1H, H-5), 3.82 (s, 3H, OMe), 3.91 (d, *J* = 10.5 Hz, 1H, H-6), 4.97 (td, *J* = 4.7, 11.4 Hz, 1H, H-4), 5.30 (m, 2H, H-7, H-8), 5.75 (d, *J* = 9.4 Hz, 1H, NH), 7.70 (m, 2H, CH_{ar}), 7.90 (d, *J* = 7.9, 1H, CH_{ar}), 8.10 (d, *J* = 6.5, 1H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 12.1 (SMe), 20.5, 21.1, 21.1 (3 OAc), 38.1 (C-3), 50.8 (C-9), 53.2, 53.5 (2C, C-5, OMe), 68.8 (C-7), 69.7 (C-4), 70.4 (C-8), 74.7 (C-6), 82.8 (C-2), 125.5, 130.4, 133.5, 135.5, 147.5 (6C, C−Ar), 167.8, 170.4 (4C, CO). ESI-MS calcd for $C_{23}H_{29}N_5O_{13}S_2$ $[M - H]^-$ 646.12; found *m*/*z* 646.56.

Methyl (Methyl 5-Methylsulfonamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (16d). Compound **15** (50.0 mg, 0.11 mmol) was dissolved in dry DCM (2.0 mL) under argon atmosphere and subsequently cooled to 0 °C. Methanesulfonylchloride (25.0 μL, 37.0 mg, 0.32 mmol), NEt_3 (45.0 μL, 33.0 mg, 0.32 mmol), and a catalytic amount of DMAP were added successively. The reaction mixture was stirred at 0 °C overnight. Then it was washed with saturated aqueous $NaHCO_3$ (2 × 5 mL) and H_2O (1 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **16d** (30 mg, 66%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.98 (t, *J* = 12.3 Hz, 1H, H-3a), 2.03 (s, 3H, SMe), 2.13, 2.17 (3 s, 9H, 3 OAc), 2.68 (dd, *J* = 4.7, 12.7 Hz, 1H, H-3b), 3.14 (s, 3H, C*H*$_3$), 3.29 (dd, *J* = 6.1, 13.4, 1H, H-9a), 3.66 (m, 2H, H-5, H-9b), 3.77 (s, 3H, OMe), 3.88 (dd, *J* = 1.6, 10.6 Hz, 1H, H-6), 4.55 (d, *J* = 9.8 Hz, 1H, NH), 5.16 (td, *J* = 4.7, 11.5 Hz, 1H, H-4), 5.26 (m, 1H, H-8), 5.48 (dd, *J* = 1.6, 7.2 Hz, 1H, H-7). ^{13}C NMR ($CDCl_3$) δ 14.3 (SMe), 20.9, 21.1, 21.4 (3 OAc), 31.7 (*C*H$_3$), 38.2 (C-3), 50.7 (C-9), 52.5 (C-5), 53.0 (OMe), 68.3, 69.6 (2C, C-7, C-8), 70.4 (C-4), 74.5 (C-6), 82.8 (C-2), 167.8, 170.3, 171.7, 172.2 (4 CO). ESI-MS calcd for $C_{18}H_{28}N_4O_{11}S_2$ $[M + Na]^+$ 563.12; found *m*/*z* 563.18.

Methyl ((2,3-Difluorobenzyl) 5-Fluoroacetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (17a). Compound **16a** (55.0 mg, 0.16 mmol) was dissolved in dry acetonitrile (2 mL). Powdered MS 3 Å (50 mg) and 2,3-difluorobenzyl alcohol (35.0 μL, 42.0 mg, 0.29 mmol) were added. The reaction mixture was stirred at rt for 1.5 h. Then the suspension was cooled to −40 °C and was subsequently treated with *N*-iodosuccinimide (35.0 mg, 0.16 mmol) and triflic acid (8.00 μL, 13.0 mg, 0.09 mmol). After 30 min, the reaction mixture was warmed to −30 °C and stirring was continued for 20 h. After warming to rt, the mixture was filtered through a pad of celite and washed with 20% $Na_2S_2O_3$ (1 × 2 mL), saturated aqueous $NaHCO_3$ (3 × 5 mL), and H_2O (1 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (1% gradient *i*PrOH in petrol ether/DCM 2:1) to yield **16a** (43 mg, 66%) as a colorless oil. $[\alpha]^{20}_D$ −0.04 (*c* 0.73, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.03 (s, 3H, OAc), 2.04 (m, 1H, H-3a), 2.16, 2.21 (2 s, 6H, 2 OAc), 2.69 (dd, *J* = 4.7, 12.9 Hz, 1H, H-3b), 3.26 (dd, *J* = 5.3, 13.5 Hz, 1H, H-9a), 3.58 (dd, *J* = 2.8, 13.5 Hz, 1H, H-9b), 3.79 (s, 3H, OMe), 4.21 (m, 2H, H-5, H-6), 4.53 (A of AB, *J* = 12.0 Hz, 1H, C*H*$_2$Ar), 4.63 (m, 2H, CH_2F), 4.83 (B of AB, *J* = 12.3 Hz, 1H, C*H*$_2$Ar), 4.95 (ddd, *J* = 4.7, 10.0, 12.1 Hz, 1H, H-4), 5.35 (m, 2H, H-7, H-8), 6.18 (dd, *J* = 3.3, 9.0 Hz, 1H, N*H*FAc), 7.11 (m, 3H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.9 (3C, OAc), 37.9 (C-3), 48.6 (C-5), 50.9 (C-9), 53.0 (OMe), 60.5 (*C*H$_2$Ar), 67.8 (C-7), 68.7 (C-8), 69.3 (C-4), 72.6 (C-6), 80.1 (*J* = 186.1 Hz, *C*H$_2$F), 98.7 (C-2), 116.9, 117.1, 125.1, 126.6 (6C, C−Ar), 168.0, 170.2, 170.7 (5C, CO). ESI-MS calcd for $C_{25}H_{29}F_3N_4O_{11}$ $[M + Na]^+$ 641.17; found *m*/*z* 641.14.

Methyl ((2,3-Difluorobenzyl) 5-Chloroacetamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (17b). Compound **16b** (63.0 mg, 0.12 mmol) was reacted with 2,3-difluorobenzyl alcohol (41.0 μL, 0.36 mmol), *N*-iodosuccinimide (42.0 mg, 0.19 mmol), and triflic acid (8.0 μL, 14.4 mg, 0.1 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient *i*PrOH in PE:DCM, 8:4) to yield **17b** (50 mg, 68%) as a colorless oil. $[\alpha]^{20}_D$ −0.01, (*c* 1.05, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.02 (m, 1H, H-3a), 2.03, 2.16, 2.21 (3 s, 9H, 3 OAc), 2.70 (dd, *J* = 4.6, 12.9 Hz, 1H, H-3b), 3.28 (dd, *J* = 5.8, 13.5 Hz, 1H, H-9a), 3.59 (dd, *J* = 3.0, 13.5 Hz, 1H, H-9b), 3.81 (s, 3H, OMe), 3.93, 4.01 (A, B of AB, *J* = 15.0 Hz, 2H, CH_2Cl), 4.11 (m, 1H, H-5), 4.24 (dd, *J* = 2.1, 10.7 Hz, 1H, H-6), 4.54, 4.83 (A, B of AB, *J* = 12.0 Hz, 2H, CH_2Ar), 4.99 (m, 1H, H-4), 5.32 (dd, *J* = 2.1, 7.8 Hz, 1H, H-7), 5.36 (m, 1H, H-8), 6.41 (d, *J* = 10.1 Hz, 1H, NH), 7.06−7.17 (m, 3H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.8, 20.9, 21.1 (3 OAc), 37.9 (C-3) 42.4 (*C*H$_2$Cl), 49.7 (C-5), 50.9 (C-9), 60.5 (*C*H$_2$Ar), 67.9, 68.1, 68.4 (3C, C-4, C-7, C-8), 72.6 (C-6), 98.7 (C-2), 117.0, 123.9, 125.1, 126.5, 149.5, 151.4 (6 C−Ar), 166.7, 168.0, 170.2, 170.3, 170.7 (5 CO). ESI-MS calcd for $C_{25}H_{29}Cl_2FN_4O_{11}$ $[M + Na]^+$ 657.14; found *m*/*z* 657.29.

Methyl ((2,3-Difluorobenzyl) 5-(*o*-Nitrotoluenesulfonamido)-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (17c). Compound **16c** (62.0 mg, 0.10 mmol) was reacted with 2,3-difluorobenzyl alcohol (30.0 μL, 38.0 mg, 0.29 mmol), *N*-iodosuccinimide (32.0 mg, 0.14 mmol), and triflic acid (7.00 μL, 12.0 mg, 0.08 mmol). The crude product was purified by chromatography on silica gel (toluene/EA, gradient 1:1 to 2:1) to yield **17c** (44 mg, 61%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.84 (t, *J* = 12.4 Hz, 1H, H-3a), 2.08, 2.17, 2.28 (3 s, 9H, 3 OAc), 2.64 (dd, *J* = 4.6, 12.8 Hz, 1H, H-3b), 3.29 (m, 1H, H-9a), 3.45 (m, 1H, H-9b), 3.73 (s, 3H, OMe), 3.79 (m, 2H, H-5, H-7), 4.12 (d, *J* = 8.8 Hz, 1H, H-6), 4.46, 4.77 (A, B of AB, *J* = 12.0 Hz, 2H, C*H*$_2$Ar), 4.91 (td, *J* = 4.6, 11.5 Hz, 1H, H-4), 5.25 (d, *J* = 7.8 Hz, 1H, H-8), 5.60 (d, *J* = 9.4 Hz, 1H, NH), 7.09 (m, 3H, CH_{ar}), 7.69 (m, 2H, CH_{ar}), 7.85 (d, *J* = 7.9 Hz, 1H, CH_{ar}), 8.07 (d, *J* = 7.8 Hz, 1H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.1 (3C, OAc), 38.1 (C-3), 51.1 (C-5), 53.1 (C-9), 53.6 (OMe), 60.5 (*C*H$_2$Ar), 68.7, 68.8 (2C, C-4, C-7), 69.8 (C-8), 73.2 (C-6), 125.5, 130.4, 133.5, 147.6 (12C, C−Ar), 168.0, 170.1, 170.3, 170.5 (4 CO). ESI-MS calcd for $C_{29}H_{31}F_2N_5O_{14}S$ $[M + Na]^+$ 766.16; found *m*/*z* 766.14.

Methyl ((2,3-Difluorobenzyl) 5-Methylsulfonamido-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (17d). Compound **16d** (56.0 mg, 0.10 mmol) was reacted with 2,3-difluorobenzyl alcohol (33.0 μL, 42.0 mg, 0.29 mmol), *N*-iodosuccinimide (35.0 mg, 0.16 mmol), and triflic acid (8.00 μL, 13.0 mg, 0.09 mmol). The crude product was purified by chromatography on silica gel (1% gradient *i*PrOH in petrol ether/DCM 2:1) to yield **17d** (37 mg, 56%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.95 (t, J = 12.5, 1H, H-3a), 2.13, 2.18 (2 s, 9H, 3 OAc), 2.61 (dd, J = 4.6, 12.7 Hz, 1H, H-3b), 3.02 (s, 3H, CH_3), 3.31 (dd, J = 6.3, 13.5 Hz, 1H, H-9a), 3.60 (dd, J = 3.3, 13.5 Hz, 1H, H-9b), 3.71 (m, 4H, H-5, OMe), 4.12 (dd, J = 1.7, 10.7 Hz, 1H, H-6), 4.54, 4.81 (A, B of AB, J = 11.8 Hz, 2H, CH_2Ar), 5.09 (m, 1H, H-4), 5.31 (m, 1H, H-8), 5.49 (dd, J = 1.7, 7.5 Hz, 1H, H-7), 7.11 (m, 3H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.9, 21.1, 21.4 (3 OAc), 38.1 (C-3), 42.3 (*C*H_3), 50.8 (C-9), 52.6, 53.0 (2C, OMe, C-5), 60.4 (*C*H_2Ar), 68.1 (C-4), 68.8 (C-7), 70.0 (C-8), 72.8 (C-6), 98.5 (C-2), 116.9, 117.0, 124.0, 125.1 (6C, C−Ar), 167.7, 170.1, 170.7, 172.1 (4 CO). ESI-MS calcd for $C_{24}H_{30}F_2N_4O_{12}S$ $[M + Na]^+$ 659.15; found m/z 659.24.

Methyl ((2,3-Difluorobenzyl) 5-Fluoroacetamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (18a). Compound **17a** (55.0 mg, 0.09 mmol) was dissolved in dry DCM (2 mL). *p*-Chlorobenzoyl chloride (45.0 μL, 62.0 mg, 0.36 mmol) and triphenylphosphine (47.0 mg, 0.18 mmol) were added. The reaction mixture was stirred at rt overnight. Afterward, the reaction mixture was washed with saturated aqueous $NaHCO_3$ (3 × 5 mL) and H_2O (1 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **18a** (31 mg, 48%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.97 (m, 1H, H-3a), 1.97, 2.09, 2.18 (3 s, 9H, 3 OAc), 2.64 (dd, J = 4.6, 12.8 Hz, 1H, H-3b), 2.94 (dd, J = 3.9, 15.0 Hz, 1H, H-9a), 3.69 (s, 3H, OMe), 4.09 (m, 1H, H-6), 4.22 (m, 2H, H-5, H-9b), 4.46 (A of AB, J = 12.0 Hz, 1H, CH_2Ar), 4.64 (m, 2H, CH_2F), 4.78 (B of AB, J = 12.0 Hz, 1H, CH_2Ar), 4.86 (m, 1H, H-4), 5.11 (dd, J = 2.0, 9.8 Hz, 1H, H-7), 5.27 (m, 1H, H-8), 6.12 (dd, J = 3.3, 10.0 Hz, 1H, N*H*FAc), 6.89 (dd, J = 4.3, 7.9 Hz, 1H, N*H*), 7.03 (m, 3H, CH_{ar}), 7.34, 7.67 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.8, 21.1 (3C, OAc), 37.9 (C-3), 38.7 (C-9), 48.7 (C-5), 52.9 (OMe), 60.5 (*C*H_2Ar), 67.8 (C-7), 68.2 (C-8), 68.7 (C-4), 72.0 (C-6), 80.0 (J = 186.0 Hz, *C*H_2F), 98.6 (C-2), 117.1, 124.0, 125.3, 126.6, 128.4, 128.8, 131.5, 132.6, 137.8 (12C, C−Ar), 166.4, 167.8, 168.3, 170.5, 170.7, 172.3 (6 CO). ESI-MS calcd for $C_{32}H_{34}ClF_3N_2O_{12}$ $[M + Na]^+$ 753.18; found m/z 753.19.

Methyl ((2,3-Difluorobenzyl) 5-Chloroacetamido-4,7,8-tri-*O*-acetyl-9-(4-chloro-benzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (18b). Compound **17b** (52.2 mg, 0.08 mmol) was reacted with *p*-chlorobenzoyl chloride (23.0 μL, 31.0 mg, 0.18 mmol) and triphenylphosphine (47.0 mg, 0.18 mmol). The crude product was purified by chromatography on silica gel (0.5% gradient of MeOH in DCM) to yield in **18b** (25.0 mg, 48%). $[\alpha]^{20}_D$ 0.04 (*c* 1.06, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.00 (m, 1H, H-3a), 2.03, 2.16, 2.25 (3 s, 9H, 3 OAc), 2.71 (m, 1H, H-3b), 3.01 (m, 1H, H-9a), 3.77 (s, 3H, OMe), 3.97 (m, 2H, CH_2Cl), 4.11 (m, 1H, H-6), 4.20 (m, 1H, H-5), 4.29 (m, 1H, H-9b), 4.54, 4.85 (A, B of AB, J = 11.9 Hz, 2H, CH_2Ar), 4.95 (m, 1H, H-4), 5.18 (m, 1H, H-7), 5.31 (m, 1H, H-8), 6.39 (m, 1H, N*H*Ac), 6.97 (m, 1H, NH), 7.06−7.15 (m, 3H, CH_{ar}), 7.40 (m, 2H, CH_{ar}), 7.74 (m, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.8, 21.2 (3C, OAc), 38.0 (C-3), 38.6 (C-9), 42.4 (*C*H_2Cl), 49.8 (C-5), 52.9 (OMe), 60.4 (*C*H_2Ar), 67.8, 68.3, 68.5 (3C, C-4, C-7, C-8), 72.1 (C-6), 98.6 (C-2), 117.0 (J = 17.5 Hz), 124.0, 125.3, 126.5, 128.4, 128.9, 132.7, 137.8 (12 C−Ar), 166.4, 166.7, 167.9, 170.5, 170.7, 172.3 (6 CO). ESI-MS calcd for $C_{32}H_{34}Cl_2F_2N_2O_{12}$ $[M + Na]^+$ 769.14; found m/z 769.34.

Methyl ((2,3-Difluorobenzyl) 5-(*o*-Nitrotoluenesulfonamido)-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (18c). Compound **17c** (44.0 mg, 0.06 mmol) was dissolved in dry DCE (2 mL). *p*-Chlorobenzoyl chloride (30.0 μL, 40.0 mg, 0.23 mmol) and triphenylphosphine (31.0 mg, 0.12 mmol) were added. The reaction mixture was stirred at rt overnight. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **18c** (29 mg, 58%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.90 (m, 1H, H-3a), 2.04, 2.14, 2.32 (3 s, 9H, 3 OAc), 2.69 (dd, J = 4.5, 12.7 Hz, 1H, H-3b), 2.92 (d, J = 15.2 Hz, 1H, H-9a), 3.75 (s, 3H, OMe), 3.91 (m, 1H, H-5), 4.12 (m, 1H, H-6), 4.38 (m, 1H, H-9b), 4.52 (A of AB, J = 11.9 Hz, 1H, CH_2Ar), 4.84 (m, 2H, H-4, CH_2Ar), 5.31 (m, 2H, H-7, H-8), 5.64 (d, J = 9.4 Hz, 1H, NH), 7.13 (m, 4H, CH_{ar}), 7.21 (m, 1H, NH), 7.40 (m, 3H, CH_{ar}), 7.72 (m, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.3, 21.2, 21.3 (3 OAc), 38.2, 38.3 (2C, C-3, C-9), 53.0 (OMe), 53.7 (C-5), 60.6 (*C*H_2Ar), 68.2, 68.4 (2C, C-7, C-8), 69.0 (C-4), 72.6 (C-6), 98.2 (C-2), 117.0, 123.9, 125.6, 128.8, 130.3, 131.5, 132.1, 132.8, 133.3 (18C, C−Ar), 166.6, 167.7, 169.9, 170.4, 172.4 (5 CO). ESI-MS calcd for $C_{36}H_{36}ClF_2N_3O_{15}S$ $[M + Na]^+$ 878.15; found m/z 878.28.

Methyl ((2,3-Difluorobenzyl) 5-Methylsulfonamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (18d). Compound **17d** (35.0 mg, 0.06 mmol) was dissolved in dry DCM (2 mL). *p*-Chlorobenzoyl chloride (28.0 μL, 38.0 mg, 0.22 mmol) and triphenylphosphine (29.0 mg, 0.11 mmol) were added. The reaction mixture was stirred at rt overnight. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **18d** (21 mg, 52%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.98 (t, J = 13.6 Hz, 1H, H-3a), 2.05, 2.10, 2.13 (3 s, 9H, 3 OAc), 2.67 (dd, J = 4.6, 12.7 Hz, 1H, H-3b), 2.99 (s, 3H, CH_3), 3.05 (m, 1H, H-9a), 3.70 (s, 3H, OMe), 3.77 (m, 1H, H-5), 4.09 (d, J = 1.5 Hz, 1H, H-6), 4.30 (ddd, J = 3.0, 8.2, 15.1 Hz, 1H, H-9b), 4.54 (A of AB, J = 12.1 Hz, 1H, CH_2Ar), 4.59 (d, J = 9.6 Hz, 1H, NH), 4.83 (B of AB, J = 11.9 Hz, 1H, CH_2Ar), 4.99 (ddd, J = 4.6, 10.4, 12.2 Hz, 1H, H-4), 5.30 (m, 1H, H-8), 5.37 (dd, J = 1.7, 9.6 Hz, 1H, H-7), 7.13 (m, 4H, CH_{ar}, NH), 7.40, 7.75 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.2, 21.2, 21.3 (3 OAc), 29.7 (C-3), 38.2, 38.8 (2C, C-9, *C*H_3), 42.3 (C-5), 52.8 (OMe), 60.5 (*C*H_2Ar), 68.1, 68.5, 69.1 (3C, C-4, C-7, C-8), 72.4 (C-6), 98.4 (C-2), 116.9, 117.1, 125.3, 128.4, 128.9, 132.1, 132.7, 137.8 (12C, C−Ar), 166.4, 167.7, 170.5, 171.6, 172.6 (5 CO). ESI-MS calcd for $C_{31}H_{35}ClF_2N_2O_{13}S$ $[M + Na]^+$ 771.15; found m/z 771.29.

Sodium ((2,3-Difluorobenzyl) 5-Fluoroacetamido-9-(4-chloro-benzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (19a). Compound **18a** (28.0 mg, 0.04 mmol) was dissolved in THF/water (2.0 mL/0.5 mL) and was reacted with LiOH (9.00 mg, 0.38 mmol). The reaction mixture was stirred at rt for 4 h. 7% HCl (aq) was added to adjust the pH to 7. The crude product was purified by reversed-phase chromatography (RP-18, 10% gradient MeOH in H_2O) followed by ion exchange chromatography (Dowex-50) and P2 size exclusion chromatography to yield **19a** (7.0 mg, 30%) as a white foam. $[\alpha]^{20}_D$ −0.73 (*c* 0.08, H_2O). ^{1}H NMR (500 MHz, D_2O) δ 1.59 (t, J = 12.2, 1H, H-3a), 2.65 (dd, J = 4.7, 12.4, 1H, H-3b), 3.31 (dd, J = 7.7, 14.0 Hz, 1H, H-9a), 3.41 (dd, J = 1.8, 8.8 Hz, 1H, H-5), 3.64 (m, 3H, H-4, H-8, H-9b), 3.77 (dd, J = 1.9, 10.5 Hz, 1H, H-6), 3.84 (m, 1H, H-7), 4.52 (A of AB, J = 11.7 Hz, 1H, CH_2Ar), 4.70 (m, 2H, CH_2Ar, CH_2F), 4.81 (d, J = 5.1 Hz, 1H, CH_2F), 7.02 (m, 3H, CH_{ar}), 7.39, 7.62 (AA′, BB′ of AA′BB′, J = 8.7 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 40.4, 42.6 (2C, C-3, C-9), 57.2 (C-5), 60.6 (*C*H_2Ar), 68.0 (C-4), 69.7 (C-7), 70.3 (C-8), 72.3 (C-6), 87.0 (*C*H_2F), 101.3 (C-2), 117.2, 125.8, 128.6, 128.8, 132.1, 137.5, 141.7 (12C, C−Ar), 173.1, 175.3, 189.7 (3 CO). HRMS calcd for $C_{25}H_{26}ClF_3N_2O_9$ $[M - H]^-$ 589.1206; found 589.1191.

Sodium ((2,3-Difluorobenzyl) 5-Chloroacetamido-9-(4-chloro-benzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (19b). Compound **18b** (21.0 mg, 0.03 mmol) was treated with LiOH (6.7 mg, 0.3 mmol) in THF/water (2.0 mL/0.5 mL). The crude product was purified by chromatography on silica gel

(0.1% gradient of H_2O in DCM/MeOH; 2:1) followed by ion exchange chromatography (Dowex 50) and P2 size exclusion chromatography to yield **19b** as a white solid (10 mg, 60%). ^{1}H NMR (500 MHz, MeOD) δ 1.58 (dd, J = 10.7, 12.1 Hz, 1H, H-3a), 2.82 (m, 1H, H-3b), 3.35 (m, 1H, H-7), 3.46 (m, 1H, H-9a), 3.68 (m, 4H, H-4, H-5, H-6, H-9b), 3.93 (m, 2H, CH_2Cl), 4.57, 4.84 (A, B of AB, J = 12.1 Hz, 2H, CH_2Ar), 7.01 (m, 3H, CH$_{ar}$), 7.35, 7.72 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH$_{ar}$). ^{13}C NMR (MeOD) δ 43.0 (C-3), 44.8 (C-9), 45.0 (*C*H$_2$Cl), 54.0 (C-5), 63.1 (*C*H$_2$Ar), 70.5, 72.2, 72.9 (3C, C-4, C-7, C-8), 74.9 (C-6), 103.8 (C-2), 119.7, 126.9, 128.4, 128.9, 131.2, 131.3, 140.1 (10 C−Ar), 170.0, 170.5, 173.11 (3 CO). HRMS calcd for $C_{25}H_{25}Cl_2F_2N_2NaO_9$ [M + Na]$^+$ 651.0701; found m/z 651.0700.

Sodium ((2,3-Difluorobenzyl) 5-(*o*-Nitrotoluenesulfonamido)-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (19c). Compound **18c** (29.0 mg, 0.03 mmol) was dissolved in THF/H_2O (2 mL/0.5 mL) and was reacted with LiOH (8.00 mg, 0.33 mmol). The crude product was purified on RP-18 (10% gradient MeOH in water) followed by ion exchange chromatography (Dowex-50) and P2 size exclusion chromatography to yield **19c** (10 mg, 40%). $[\alpha]^{20}_D$ −0.35 (c 0.10, H_2O). ^{1}H NMR (500 MHz, D_2O) δ 1.45 (t, J = 12.2 Hz, 1H, H-3a), 2.52 (dd, J = 4.5, 12.3 Hz, 1H, H-3b), 3.16 (t, J = 9.8 Hz, 1H, H-5), 3.43 (m, 2H, H-4, H-9a), 3.60 (dd, J = 2.9, 14.2 Hz, 1H, H-9b), 3.67 (m, 2H, H-6, H-8), 3.74 (dd, J = 1.1, 8.9 Hz, 1H, H-7), 4.50 (A of AB, J = 11.7 Hz, 1H, CH_2Ar), 4.65 (m, 1H, CH_2Ar), 7.04 (m, 3H, CH$_{ar}$), 7.42 (AA′ of AA′BB′, J = 8.5 Hz, 2H, CH$_{ar}$), 7.60 (m, 4H, CH$_{ar}$), 7.98 (BB′ of AA′BB′, J = 7.6 Hz, 2H, CH$_{ar}$). ^{13}C NMR (D_2O) δ 40.4 (C-3), 42.4 (C-9), 56.5 (C-5), 60.5 (*C*H$_2$Ar), 69.4 (C-7), 69.5 (C-4), 70.3 (C-8), 73.8 (C-6), 101.2 (C-2), 117.1, 124.2, 125.8, 128.7, 130.2, 132.1, 132.5, 137.5, 146.8 (18C, C−Ar), 170.0, 173.3 (2 CO). HRMS calcd for $C_{29}H_{27}ClF_2N_3NaO_{12}S$ [M + Na]$^+$ 760.0767; found m/z 760.0775.

Sodium ((2,3-Difluorobenzyl) 5-Methylsulfonamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (19d). Compound **18d** (21.0 mg, 0.03 mmol) was dissolved in THF/H_2O (2 mL/0.5 mL) and was reacted with LiOH (7.00 mg, 0.28 mmol). The crude product was purified on RP-18 (10% gradient MeOH in water) followed by ion exchange chromatography (Dowex-50) and P2 size exclusion chromatography to yield **19d** (10 mg, 59%). $[\alpha]^{20}_D$ −0.44 (c 0.07, H_2O). ^{1}H NMR (500 MHz, D_2O) δ 1.56 (t, J = 12.2 Hz, 1H, H-3a), 2.62 (dd, J = 4.6, 12.4 Hz, 1H, H-3b), 3.02 (s, 3H, CH_3), 3.23 (t, J = 10.1 Hz, 1H, H-5), 3.40 (dd, J = 7.8, 14.7 Hz, 1H, H-9a), 3.48 (td, J = 4.6, 11.9 Hz, 1H, H-4), 3.66 (m, 3H, H-6, H-8, H-9b), 3.75 (d, J = 8.7 Hz, 1H, H-7), 4.51 (A of AB, J = 11.8 Hz, 1H, CH_2Ar), 4.66 (m, 1H, CH_2Ar), 7.02 (m, 3H, CH$_{ar}$), 7.40, 7.63 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH$_{ar}$). ^{13}C NMR (D_2O) δ 40.9, 41.3 (2C, C-3, C-9), 42.5 (*C*H$_3$), 55.5 (C-5), 60.6 (*C*H$_2$Ar), 68.6 (C-7), 69.3 (C-4), 70.3 (C-8), 73.2 (C-6), 101.2 (C-2), 112.4, 117.2, 124.4, 125.8, 128.7, 128.8, 137.5 (12C, C−Ar), 173.1, 187.7 (2 CO). HRMS calcd for $C_{24}H_{26}ClF_2N_2NaO_{10}S$ [M + Na]$^+$ 653.0760; found m/z 653.0759.

Sodium ((2,3-Difluorobenzyl) 5-Methoxyacetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (19e). Compound **18b** (10 mg, 0.01 mmol) was treated with 10% aqueous NaOH (0.3 mL) in MeOH (1.5 mL) at rt for 4 h. The crude product was purified by chromatography on RP-18 (5% gradient of MeOH in H_2O) to yield **19e** (2.3 mg, 19%) and **19b** (1.7 mg, 21%) as white solids. $[\alpha]^{20}_D$ −19.1 (c 0.46, MeOH). ^{1}H NMR (500 MHz, D_2O) δ 1.67 (t, J = 12.2 Hz, 1H, H-3a), 2.73 (dd, J = 4.6, 12.4 Hz, 1H, H-3b), 3.28 (s, 3H, OMe), 3.41−3.56 (m, 2H, H-7, H-9a), 3.62−3.79 (m, 3H, H-4, H-8, H-9b), 3.82−3.89 (m, 2H, H-5, H-6), 3.95, 3.98 (A, B of AB, J = 15.6 Hz, 2H, CH_2OMe), 4.62, 4.78 (A, B of AB, J = 11.7 Hz, 2H, CH$_2$Ar), 7.00−7.22 (m, 3H, CH$_{ar}$), 7.49, 7.71 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH$_{ar}$). ^{13}C NMR (D_2O) δ 40.4 (C-3), 42.4 (C-9), 51.5 (C-5), 58.9 (OMe), 60.6 (CH$_2$Ar), 67.9 (C-4), 69.6 (C-7), 70.2 (C-8), 70.8 (*C*H$_2$OMe), 72.4 (C-6), 101.2 (C-2), 110.0, 117.1, 117.2, 124.4, 125.9, 128.7, 128.8, 132.1 (12C, C−Ar), 173.0, 173.5 (3C, CO). HRMS calcd for $C_{26}H_{28}ClF_2N_2O_{10}Na$ [M + Na]$^+$ 647.1195; found m/z 647.5573.

Sodium ((2,3-Difluorobenzyl) 5-Cyclopropylamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (19f). Compound **23f** (31.0 mg, 0.04 mmol) was dissolved in THF/water (2.0 mL/0.5 mL) and was reacted with LiOH (10.0 mg, 0.42 mmol). The crude product was purified on RP-18 (10% gradient MeOH in H_2O) followed by ion exchange chromatography (Dowex-50) and P2 size exclusion chromatography to yield **19f** (11 mg, 44%). $[\alpha]^{20}_D$ −0.1 (c 0.26, H_2O). ^{1}H NMR (500 MHz, D_2O) δ 0.59 (m, 2H, CH_2), 0.70 (m, 2H, CH_2), 1.44 (m, 1H, CH), 1.56 (t, J = 11.8 Hz, 1H, H-3a), 2.64 (dd, J = 3.5, 11.8 Hz, 1H, H-3b), 3.37 (d, J = 8.2 Hz, 1H, H-7), 3.47 (m, 1H, H-9a), 3.63 (m, 4H, H-5, H-6, H-8, H-9b), 4.52, 4.69 (A, B of AB, J = 11.6 Hz, 2H, CH_2Ar), 7.04 (m, 3H, CH$_{ar}$), 7.40, 7.63 (AA′, BB′ of AA′BB′, J = 8.1 Hz, 4H, CH$_{ar}$). ^{13}C NMR ($CDCl_3$) δ 6.8, 7.0 (2C, *C*H$_2$), 14.1 (*C*H), 40.5, 42.3 (2C, C-3, C-9), 51.9 (C-5), 60.5 (*C*H$_2$Ar), 68.0 (C-4), 69.4 (C-7), 70.1 (C-8), 72.8 (C-6), 101.2 (C-2), 117.1, 124.4, 125.9, 128.7, 132.1, 137.6 (12C, C−Ar), 170.0, 173.1, 178.3 (3 CO). HRMS calcd for $C_{27}H_{28}ClF_2N_2O_9$ [M − H]$^-$ 597.1457; found 597.1454.

Sodium ((2,3-Difluorobenzyl) 5-Cyclobutylamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (19g). Compound **23g** (44.0 mg, 0.06 mmol) was dissolved in THF/water (2.0 mL/0.5 mL) and was reacted with LiOH (14.0 mg, 0.58 mmol). The crude product was purified on RP-18 (10% gradient MeOH in H_2O) followed by ion exchange chromatography (Dowex-50) and P2 size exclusion chromatography to yield **19g** (17 mg, 49%). $[\alpha]^{20}_D$ −0.2 (c 0.11, H_2O). ^{1}H NMR (500 MHz, D_2O) δ 1.55 (m, 1H, H-3a), 1.85 (m, 6H, CH_2), 2.63 (dd, J = 3.9, 12.2 Hz, 1H, H-3b), 2.99 (quint, J = 8.8 Hz, 1H, CH), 3.32 (d, J = 8.6 Hz, 1H, H-9a), 3.61 (m, 6H, H-4, H-5, H-6, H-7, H-8, H-9b), 4.52, 4.68 (A, B of AB, J = 11.4 Hz, 2H, CH_2Ar), 7.05 (m, 3H, CH$_{ar}$), 7.40, 7.63 (AA′, BB′ of AA′BB′, J = 7.5 Hz, 4H, CH$_{ar}$). ^{13}C NMR (D_2O) δ 17.4, 24.5 (3C, *C*H$_2$), 39.2 (*C*H), 40.5 (C-3), 42.1 (C-9), 51.6 (C-5), 60.5 (*C*H$_2$Ar), 67.8 (C-4), 69.3 (C-7), 70.1 (C-8), 72.7 (C-6), 110.0 (C-2), 115.6, 123.3, 125.9, 128.8, 150.9 (12C, C−Ar), 170.0, 175.8. 179.7 (3 CO). ESI-MS calcd for $C_{28}H_{30}ClF_2N_2NaO_9$ [M + Na]$^+$ 657.1403; found m/z 657.1401.

Methyl (Methyl 5-*tert*-Butyloxycarbonylamino-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-tride-oxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (20). Compound **14** (167 mg, 0.30 mmol) was dissolved in dry DCE (5 mL). *p*-Chlorobenzoyl chloride (150 μL, 210 mg, 1.19 mmol) and triphenylphosphine (156 mg, 0.59 mmol) were added. The reaction mixture was stirred at rt overnight. Afterward, the reaction mixture was washed with saturated aqueous $NaHCO_3$ (3 × 5 mL) and H_2O (1 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **20** (95 mg, 48%) as a white foam. ^{1}H NMR (500 MHz, CD_2Cl_2) δ 1.39 (s, 9H, C(CH_3)$_3$), 1.92 (s, 3H, SMe), 2.01 (s, 6H, 2 OAc), 2.12 (m, 1H, H-3a), 2.23 (s, 3H, OAc), 2.56 (dd, J = 4.7, 13.8 Hz, 1H, H-3b), 3.00 (td, J = 3.2, 15.2 Hz, 1H, H-9a), 3.79 (m, 4H, OMe, H-5), 4.23 (d, J = 10.6 Hz, 1H, H-6), 4.41 (ddd, J = 3.1, 8.8, 14.4 Hz, 1H, H-9b), 4.52 (dd, J = 2.9, 10.0 Hz, 1H, N*H*Ac), 5.09 (m, 1H, H-8), 5.16 (td, J = 4.6, 11.0 Hz, 1H, H-4), 5.33 (m, 1H, H-7), 7.23 (d, J = 3.9 Hz, 1H, NH), 7.54, 8.08 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH$_{ar}$). ^{13}C NMR (CD_2Cl_2) δ 11.7 (SMe), 21.2, 21.3, 21.4 (3 OAc), 28.5 (C(*C*H$_3$)$_3$), 37.8 (C-3), 38.4 (C-9), 51.5 (C-5), 53.3 (OMe), 68.9 (C-7), 70.0 (C-4), 70.6 (C-8), 71.5 (C-6), 80.5 (C-2), 85.3 (*C*(CH$_3$)$_3$), 129.3, 132.4, 138.0, 141.8 (6C, C−Ar), 155.7 (CONH), 166.7, 168.6, 170.5, 170.7, 172.4 (5 CO). ESI-MS calcd for $C_{29}H_{39}ClN_2O_{12}S$ [M + Na]$^+$ 697.18; found m/z 697.25.

Methyl (Methyl 5-Amino-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (21). Compound **20** (95.0 mg, 0.14 mmol) was dissolved in 4 M PhOH (in DCM; 4 mL) and 4 M TMSCl

(in DCM; 2 mL). The reaction mixture was stirred at rt for 2 h. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 8:1) to yield **21** as a white foam (51 mg, 63%). ^{1}H NMR (500 MHz, CD_2Cl_2) δ 1.95 (s, 3H, SMe), 1.99 (dd, J = 5.7, 14.0 Hz, 1H, H-3a), 2.04, 2.06, 2.19 (3 s, 9H, 3 OAc), 2.57 (dd, J = 4.7, 13.7 Hz, 1H, H-3b), 2.62 (t, J = 10.0 Hz, 1H, H-5), 3.34−3.42 (m, 1H, H-9a), 3.76 (s, 3H, OMe), 4.06 (dd, J = 1.4, 10.0 Hz, 1H, H-6), 4.17 (ddd, J = 3.3, 7.0, 15.0 Hz, 1H, H-9b), 4.98 (td, J = 4.7, 11.4 Hz, 1H, H-4), 5.18 (dt, J = 3.7, 7.5 Hz, 1H, H-8), 5.61 (dd, J = 1.5, 7.5 Hz, 1H, H-7), 6.90 (m, 2H, NH_2), 7.43, 7.74 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}). ^{13}C NMR (CD_2Cl_2) δ 11.8 (SMe), 21.3, 21.4, 21.4 (3 OAc), 37.0 (C-3), 39.4 (C-9), 52.2 (C-5), 53.3 (OMe), 69.5 (C-7), 71.3 (C-8), 73.0 (C-4), 73.8 (C-6), 84.7 (C-2), 129.1, 129.3, 130.1, 133.5 (6C, C−Ar), 166.3, 168.2, 170.3, 170.4, 171.4 (5 CO). ESI-MS calcd for $C_{24}H_{31}ClN_2O_{10}S$ $[M + Na]^+$ 597.13; found m/z 597.14.

Methyl (Methyl 5-Cyclopropylamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (22f). Compound **21** (74.0 mg, 0.16 mmol) was dissolved in dry DCM (2.0 mL) under argon atmosphere. Cyclopropanoyl chloride (24.0 μL, 28.0 mg, 0.27 mmol), NEt_3 (37.0 μL, 27.0 mg, 0.27 mmol), and a catalytic amount of DMAP were added successively. The reaction mixture was stirred at rt for 3.5 h. Then it was washed with saturated aqueous $NaHCO_3$ (3 × 5 mL) and H_2O (1 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **22f** (43 mg, 75%). $[\alpha]^{20}_D$ 0.48 (c 2.16, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.70 (m, 2H, CH_2), 0.91 (m, 2H, CH_2), 1.22 (m, 1H, CH), 2.01 (m, 4H, H-3a, SMe), 2.10, 2.12, 2.20 (3 s, 9H, 3 OAc), 2.71 (dd, J = 4.6, 12.7 Hz, 1H, H-3b), 2.86 (m, 1H, H-9a), 3.73 (dd, J = 2.0, 10.7 Hz, 1H, H-6), 3.76 (s, 3H, OMe), 4.24 (q, J = 10.5 Hz, 1H, H-5), 4.36 (ddd, J = 2.9, 8.7, 11.6 Hz, 1H, H-9b), 4.84 (td, J = 4.6, 11.6 Hz, 1H, H-4), 5.12 (dd, J = 2.0, 10.1 Hz, 1H, H-7), 5.25 (dt, J = 2.7, 10.1 Hz, 1H, H-8), 5.42 (d, J = 10.3 Hz, 1H, 5-NH), 7.18 (dd, J = 4.0, 8.7 Hz, 1H, NH), 7.39, 7.75 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 7.4 (2C, CH_2), 12.1 (CH), 14.6 (SMe), 21.1 (3C, 3 OAc), 37.8, 38.2 (2C, C-3, C-9), 49.4 (C-5), 52.9 (OMe), 67.9, 68.2 (2C, C-7, C-8), 69.7 (C-4), 73.9 (C-6), 82.8 (C-2), 128.8, 129.4, 132.7, 137.7 (6C, C−Ar), 166.1, 167.7, 170.3, 171.2, 172.4, 173.7 (6 CO). ESI-MS calcd for $C_{28}H_{35}ClN_2O_{11}S$ $[M + Na]^+$ 665.17; found m/z 665.06.

Methyl (Methyl 5-Cyclobutylamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-2-thio-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (22g). Compound **21** (60 mg, 0.1 mmol) was dissolved in dry DCM (2.4 mL). Cyclobutanecarbonyl chloride (36 μL, 37 mg, 0.3 mmol), NEt_3 (44 μL, 32 mg, 0.3 mmol), and a catalytic amount of DMAP were added successively. The reaction mixture was stirred at rt overnight. Then it was washed with saturated aqueous $NaHCO_3$ (3 × 5 mL) and H_2O (1 × 5 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (EA:PE, gradient 1:1 to 2:1) to yield **22g** (27 mg, 39%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.00 (m, 4H, SMe, H-3a), 2.03 (s, 3H, OAc), 2.08 (m, 2H, CH_2), 2.13 (s, 3H, OAc), 2.18 (m, 4H, CH_2), 2.27 (s, 3H, OAc), 2.71 (dd, J = 4.6, 12.7 Hz, 1H, H-3b), 2.86 (m, 2H, CH, H-9a), 3.71 (dd, J = 2.1, 10.8 Hz, 1H, NH), 3.77 (s, 3H, OMe), 4.20 (t, J = 10.4 Hz, 1H, H-5), 4.37 (ddd, J = 2.9, 8.8, 15.2 Hz, 1H, H-9b), 4.82 (td, J = 4.6, 11.6 Hz, 1H, H-4), 5.08 (d, J = 10.3 Hz, 1H, H-6), 5.27 (m, 2H, H-7, H-8), 7.13 (dd, J = 4.0, 8.6 Hz, 1H, 5-NH), 7.38, 7.74 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 12.1 (SMe), 18.2 (CH_2), 20.9 (3C, OAc), 24.9 (2C, CH_2), 37.8, 38.2 (2C, C-3, C-9), 39.8 (CH), 49.2 (C-5), 53.0 (OMe), 68.0, 68.1, 69.6 (3C, C-4, C-7, C-8), 73.8 (C-4), 82.8 (C-2), 128.4, 132.7, 137.7 (6C, C−Ar), 166.1, 167.7, 170.3, 172.6, 175.0 (6C, CO). ESI-MS calcd for $C_{29}H_{37}ClN_2O_{11}S$ $[M + Na]^+$ 679.18; found m/z 679.11.

Methyl ((2,3-Difluorobenzyl) 5-Cyclopropylamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (23f). Compound **22f** (37.0 mg, 0.06 mmol) was reacted with 2,3-difluorobenzyl alcohol (18.0 μL, 23.0 mg, 0.16 mmol), *N*-iodosuccinimide (20.0 mg, 0.09 mmol), and triflic acid (4.00 μL, 7.00 mg, 0.05 mmol). The crude product was purified by chromatography on silica gel (1% gradient *i*PrOH in petrol ether/DCM 2:1) to yield **23f** (31 mg, 72%). $[\alpha]^{20}_D$ 0.23 (c 1.73, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.79 (m, 2H, CH_2), 1.00 (m, 2H, CH_2), 1.29 (m, 1H, CH), 2.08 (m, 1H, H-3a), 2.09, 2.21, 2.29 (3 s, 9H, 3 OAc), 2.72 (dd, J = 4.5, 12.8 Hz, 1H, H-3b), 2.98 (m, 1H, H-9a), 3.82 (s, 3H, OMe), 4.11 (m, 1H, H-6), 4.34 (m, 1H, H-5), 4.41 (m, 1H, H-9b), 4.59, 4.89 (A, B of AB, J = 12.0 Hz, 2H, CH_2Ar), 4.95 (m, 1H, H-4), 5.21 (dd, J = 1.8, 10.0 Hz, 1H, H-7), 5.36 (m, 1H, H-8), 5.49 (d, J = 10.2 Hz, 1H, 5-NH), 7.19 (m, 4H, NH, CH_{ar}), 7.47, 7.84 (AA′, BB′, J = 8.5 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 7.1, 7.4 (2C, 2 CH_2), 14.6 (SMe), 20.8, 21.2, 21.3 (3 OAc), 25.4 (CH), 38.0, 38.4 (2C, C-3, C-9), 49.5 (C-5), 52.8 (OMe), 60.5 (CH_2Ar), 67.9, 68.4, 68.9 (3C, C-4, C-7, C-8), 72.5 (C-6), 98.6 (C-2), 115.8, 117.1, 123.9, 125.4, 126.7, 128.5, 132.7, 137.7 (12C, C−Ar), 166.2, 168.0, 170.3, 171.2, 172.4, 173.8 (6 CO). ESI-MS calcd for $C_{34}H_{37}ClF_2N_2O_{12}$ $[M + Na]^+$ 761.20; found m/z 761.16.

Methyl ((2,3-Difluorobenzyl) 5-Cyclobutylamido-4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid)onate (23g). Compound **22g** (53.0 mg, 0.08 mmol) was reacted with 2,3-difluorobenzyl alcohol (25.0 μL, 33.0 mg, 0.23 mmol), *N*-iodosuccinimide (27.0 mg, 0.12 mmol), and triflic acid (6.00 μL, 10.0 mg, 0.07 mmol). The crude product was purified by chromatography on silica gel (1% gradient *i*PrOH in petrol ether/DCM 2:1) to yield **23g** (44 mg, 72%). $[\alpha]^{20}_D$ 0.23 (c 1.7, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.85 (m, 1H, H-3a), 1.99 (s, 3H, OAc), 2.04 (m, 4H, 2 CH_2), 2.13 (s, 3H, OAc), 2.18 (m, 2H, CH_2), 2.27 (s, 3H, OAc), 2.63 (dd, J = 4.5, 12.7 Hz, 1H, H-3b), 2.84 (quint, J = 10.0 Hz, 1H, CH), 2.91 (dt, J = 3.5, 15.0 Hz, 1H, H-9a), 3.73 (s, 3H, OMe), 4.03 (d, J = 12.5 Hz, 1H, H-6), 4.22 (q, J = 10.4 Hz, 1H, H-5), 4.33 (ddd, J = 2.8, 8.6, 15.1 Hz, 1H, H-9b), 4.51 (A of AB, J = 11.8 Hz, 1H, CH_2Ar), 4.82 (m, 2H, H-4, CH_2Ar), 5.10 (dd, J = 1.7, 9.9 Hz, 1H, H-7), 5.17 (d, J = 10.4 Hz, 1H, 5-NH), 5.26 (m, 1H, H-8), 5.36 (d, J = 10.2 Hz, 1H, 5-NH), 7.09 (m, 3H, CH_{ar}), 7.19 (dd, J = 4.9, 8.3 Hz, 1H, NH), 7.37, 7.73 (AA′, BB′ of AA′BB′, J = 8.4 Hz, 4H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 18.1 (2C, CH_2), 21.2 (3C, OAc), 25.3 (CH_2), 37.9 (C-3), 38.3 (C-9), 39.7 (CH), 49.1 (C-5), 52.8 (OMe), 60.3 (CH−Ar), 67.8, 68.2, 68.8 (3C, C-4, C-7, C-8), 72.3 (C-6), 98.5 (C-2), 116.4, 117.1, 123.7, 125.3, 128.7, 132.5, 137.6 (12C, C−Ar), 167.9, 171.1, 172.4, 175.0 (6C, CO). ESI-MS calcd for $C_{35}H_{39}ClF_2N_2O_{12}$ $[M + Na]^+$ 775.22; found m/z 775.25.

Hapten Inhibition Assays with MAG_{d1-3}-Fc. Murine MAG_{d1-3}-Fc was affinity purified from CHO-Lec 3.2.8.1 cell culture supernatant as described before,[40] dialyzed against 10 mM phosphate buffer pH 7.4, sterile filtered, and stored at 4 °C. The purified protein is stable for several months. The protein was analyzed by an ELISA and binding assay with immobilized fetuin. Inhibition assays for MAG were performed as described previously.[26,30,40] In brief, fetuin was immobilized in microtiter plates and binding of MAG-Fc was determined in the presence of seven to eight different concentrations for each inhibitor using alkaline phosphatase-labeled anti-Fc antibodies. The half-maximal inhibitory concentrations were determined from corresponding binding curves and used to calculate relative inhibitory concentrations (rIC_{50}).

SPR Analysis. The SPR measurements were performed on a Biacore 3000 surface plasmon resonance based optical biosensor (Biacore AB, Sweden). Sensor chips (CM5 and CM4), immobilization kits, maintenance supply and HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) were purchased from Biacore AB (HBS-EP ready-to-use; degassed and filtered). CM5 (CM4, respectively) chips were preconditioned prior to usage by injecting a series of

conditioning solutions. A flow rate of 50 μL/min was used and 2 $\times$ 20 μL of 50 mM NaOH, 10 mM HCl, 0.1% SDS, and 100 mM H_3PO_4 were injected. The carboxy groups on the CM5 (CM4) chip were activated for 10 min with a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.1 M 3-(*N*,*N*-dimethylamino)propyl-*N*-ethylcarbodiimide (EDC) at a flow rate of 10 μL/min. Protein A (P6031) was purchased from Sigma. A sample and a reference surface were prepared sequentially or in parallel. For immobilizing protein A, a stock solution (1 mg/mL in 50 mM phosphate buffer, pH 7.0) was diluted in 10 mM sodium acetate, pH 5.0 to obtain a concentration of 30 μg/mL. This solution was then injected over the activated surface for 10 min at a flow rate of 10 μL/min. Protein A densities around 4000 RU and 5000 RU were achieved. Flow cells were blocked with a 10 min injection of 1 M ethanolamine, pH 8.0. For capturing, MAG_{d1-3}-Fc solution (expressed and purified as described[40]) was diluted to a 30−40 μg/mL concentration using HBS-EP. Afterward, MAG_{d1-3}-Fc was injected at a flow rate of 1 μL/min for 10 min. The surface was equilibrated overnight at a flow rate of 5 μL/min, achieving densities around 2000 RU. 10-fold dilution series were freshly prepared in eluent buffer immediately before use. All binding experiments were conducted at 25 °C (except thermodynamic measurements) at a flow rate of 20 μL/min. The samples were injected over 1 min, followed by 1 min dissociation. Each sample was measured with a duplicate of one concentration, using a randomized concentration order. Several buffer samples were injected before the first concentration, and one blank between each concentration, which were used for the double blank referencing during data processing. Double referencing was applied to correct for bulk effects and other systematic artifacts. Data processing and equilibrium binding constant determinations were accomplished with Scrubber (BioLogic Software, Version 1.1 g or 2.0a). Kinetic data were simultaneously fit using the nonlinear regression program Clamp or Scrubber 2.0a.

Stability Test. To dermine the stability of a compound in the central nervous system, an artificial cerebrospinal fluid (aCSF) was prepared based on published data.[41] The following concentrations (all in mM/L) were used: sodium 140, chlorine 125, hydrogen carbonate 22.5, potassium 2.9, calcium 1.15, magnesium 1, urea 4.16, and glucose 3.2. Because the composition of proteins in the CSF is comparable to the serum but at a lower concentration (the ratio of liquor protein to serum protein is 4×10^{-3}),[59] 0.4% v/v of human plasma (Sigma-Aldrich) was added and the pH was adjusted to 7.3. Then 100 μM solutions of the compound were prepared and shaken at 37 °C and 300 rpm on an Eppendorf-Thermomixer Comfort. Samples were withdrawn after 0, 30, 60, 120, 180 min and 20 h, respectively. The value assigned to every time point was the average of a triplicate measurement. The quantification of the samples was performed on a Agilent 1100 series HPLC instrument with a UV-DAD spectrometer using the ChemStation software.

log$D_{7.3}$ Determination. Two similar ratios of octanol to buffer were chosen according to the expected logD value, whereas every ratio was measured as a triplicate. Phosphate buffer at pH 7.3 was prepared and shaken overnight together with octanol in order to mutually saturate the two phases. Upon separation of the two layers, the buffer phase was withdrawn and mixed with an analyte stock solution in DMSO to yield a final concentration of 10^{-4} M. Both phases were transferred to a PCR plate, which was covered with aluminum foil (Axygen PCR-AS-200) and shaken for 1 h at 1200 rpm and 25 °C on a PHMP-4 instrument (Grant-bio). After 2.5 h at room temperature, the aqueous phases were transferred to microvials, centrifuged for 30 s, and analyzed by HPLC (Agilent 1100 series). The values were accepted if the mean values of the two ratios did not differ by more than 0.1 unit.

BBB-PAMPA.[42] Consumables (system solution, P/N 110151; brain sink buffer, P/N 110674; BBB-1 lipid solution, P/N 110672; preloaded PAMPA sandwich with stirring devices, P/N 110 212) were purchased from pION. Each donor compartment of the preloaded PAMPA plate was filled with 200 μL of pION's system solution at pH 7.4, containing the analytes at a concentration of 50 μM. Then 150 μL of the same solution were transferred to an UV-plate (UV-Star, Greiner Bio-one) and UV spectra were recorded as reference on a SpectraMax instrument (Molecular Devices). The filter membranes of the acceptor compartments were impregnated with 5 μL of BBB-1 lipid solution and each compartment was filled with 200 μL of brain sink buffer. The system was assembled and individual stirring of the wells was induced by pION's GutBox to yield an unstirred water layer thickness of 40 μm. After 30 min, the UV data of the acceptor and the donor plate were acquired on a SpectraMax instrument (Molecular Devices) and analyzed by the PAMPA Evolution command software (version 3.4, pION).

NMR. Shigemi NMR tubes were used to reduce the sample volume needed for measurement to 250 μL. MAG_{d1-3}-Fc protein was diluted from a stock solution of 1 mg/mL by a factor of 2 using 99.8% D_2O (Armar Chemicals). Following dilution, the 0.5 mg/mL MAG_{d1-3}-Fc was in a solvent of 50% D_2O and 50% H_2O, with 0.01% NaN_3 with a buffer of 5 mM PBS. Stock solutions of **4** were prepared in D_2O at 100, 50, and 20 mM and added to the NMR samples containing MAG_{d1-3}-Fc for both the titration curve and competition experiments. Stock solutions of **13f** and **25** were prepared in D_2O at 5 mM to add to the NMR samples containing MAG_{d1-3}-Fc for the competition experiments. All NMR experiments were carried out at 300 K on a Bruker DRX500 spectrometer, equipped with *Z*-gradient SEI probe. The pulse sequence used for the selective inversion recovery experiments began with a selective 25 ms I-Burp-1[60] 180° pulse applied to the *para*-hydrogen of the benzamide group of compound **4**. This proton does not overlap with any other resonances of **13f** and **25**. A further benefit of the *para*-hydrogen of the benzamide group of compound **4** was that its resonance frequency was sufficiently different from the water resonance, thus avoiding complications due to radiation damping.[61] Following the selective inversion pulse, a 1 ms gradient pulse was applied to dephase any residual transverse magnetization. The gradient pulse was followed by a variable delay to allow for the recovery of longitudinal magnetization. The delay was followed by a DPFGSE water suppression sequence to suppress the magnetization from the 50% H_2O.[61]

For each selective inversion recovery time measurement (sT1), 10 experiments were performed. These experiments consisted of increasing delays following the selective inversion pulse and gradient of 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, and 10 s. Then 32 scans, preceded by eight dummy scans, were measured for the concentrations of compound **4** of 500 μM and 1 mM. Sixteen scans, preceded by eight dummy scans, were measured for the concentrations of compound **4** of 2, 4, and 7 mM. For the competitive experiments with 25 μM of either **13f** or **25** added to 500 μM of **5**, 32 scans, preceded by eight dummy scans, were measured. A delay of 20 s following the measurement of each transient was inserted to allow the magnetization to return to equilibrium. Prior to the measurement upon addition of either **13f** or **25**, a 1 h incubation time for equilibration was allowed. The NMR data were analyzed using XWINNMR version 3.5 operating on a PC running under Linux OS. The spectra were apodized with an exponential decay function with 2 Hz line broadening. The inversion recovery data, as well as the one-site binding model, were fit using Prism 4 (GraphPad Software Inc., San Diego, CA).

Acknowledgment. We thank the Volkswagen Foundation, the Swiss National Science Foundation, the German Federal Ministry for Education and Research (BMBF, project 031632A), and the Tönjes−Vagt Foundation (project XXI) for their support of this work.

Supporting Information Available: Surface plasmon resonance assay, structure of compounds implemented additionally

in the Biacore−Hapten assay correlation, HRMS data, HPLC traces, and ^{1}H spectra of target compounds **13a−f**, **19a−g**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Kinetic and thermodynamic properties of MAG antagonists

The influence of 4- and 5-substituents on the kinetic behaviour of MAG antagonists was investigated. Furthermore, the thermodynamic properties of the most promising compound were elucidated.

This part was accepted by the *Journal of Carbohydrate Research:*
S. Mesch, K. Lemme, H. Koliwer-Brandl, D. S. Strasser, O. Schwardt, S. Kelm, B. Ernst, *in press.*

Work performed by Stefanie Mesch:
Synthesis of MAG-Antagonists and performance and evaluation of the Biacore assay

ARTICLE IN PRESS

Carbohydrate Research xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Kinetic and thermodynamic properties of MAG antagonists

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ARTICLE INFO

Article history:
Received 21 January 2010
Received in revised form 4 March 2010
Accepted 9 March 2010
Available online xxxx

Keywords:
Carbohydrate mimetics
Siglecs
Myelin-associated glycoprotein (MAG)
Thermodynamics of carbohydrate–protein interactions
Kinetics of carbohydrate–lectin interactions

ABSTRACT

Paraplegia is caused by injuries of the central nervous system (CNS) and especially young people suffer from these severe consequences as, for example, the loss of motor functions. The lack of repair of the injured nerve strands originates from the inhibitory environment for axon regeneration in the CNS. Specific inhibitory proteins block the regrowth of nerve roots. One of these neurite outgrowth inhibitors is the myelin-associated glycoprotein (MAG), which is a member of the Siglec family (sialic acid-binding immunoglobulin-like lectin). In previous studies, we identified potent small molecule MAG antagonists. In this communication, we report new neuraminic acid derivatives modified in the 4- and 5-position, and the influence of various structural modifications on their kinetic and thermodynamic binding properties.

1. Introduction

Paraplegia is caused by injuries of the central nervous system (CNS). A therapy for full regeneration of injured nerve strands is not yet available. The lack of regeneration originates from the inhibitory environment in the CNS,[1,2] that is, specific inhibitors on residual myelin and on astrocytes, which are recruited to the site of injury.[3–5] In the last decade, several inhibitor proteins have been identified, one of them being the myelin-associated glycoprotein (MAG).[6] MAG is a transmembrane glycoprotein, belonging to the Siglec family (sialic acid-binding immunoglobulin-like lectin).[7,8] On the surface of neurons, MAG interacts with two classes of targets: proteins of the Nogo receptor family[9,10] and gangliosides, primarily the gangliosides GD1a and GT1b.[11–14] Although the relative role of Nogo receptors and gangliosides as MAG ligands has yet to be resolved, in some systems, neurite outgrowth can be initiated by sialidase treatment, suggesting that the sialic acid-mediated interactions of MAG predominantly contribute to the inhibitory process.[15] Therefore, blocking MAG with potent glycomimetic antagonists may be a valuable therapeutic approach to enhance axon regeneration. Based on the best known natural ligand of MAG identified to date, the ganglioside GQ1bα (Fig. 1), different series of antagonists have been developed.[16–21]

With neuraminic acid derivatives such as **1**,[16] Kelm et al. reported a remarkable simplification of the relevant tetrasaccharide binding epitope of GQ1bα. Further reported modifications are related to lipophilic interactions. Thus, antagonists with a lipophilic core, for example, the biphenyl derivatives **2**[18] or a lipophilic replacement of the α-(2→6)-linked Neu5Ac, for example, **3**[19] were synthesized (Fig. 1).

The concept of drug discovery is based upon selectively addressing particular biological targets preferably by low molecular weight compounds. In vitro determined drug–target interactions are classically rated in terms of binding parameters such as IC_{50}'s and K_D's. An alternative perspective on drug optimization is the residence time of the drug–target binary complex,[22] as quantified by the dissociation half-life ($t_{1/2}$). Potential advantages of a long residence time are extended duration of the pharmacological effect and target selectivity.[22,23] Especially in the field of carbohydrate–lectin interactions, this is a crucial point to address. As a result of the shallow and water accessible binding sites of lectins, carbohydrates bind with only low affinity and show very fast dissociation off-rates, leading to $t_{1/2}$ in the range of seconds. Examples

Abbreviations: AIBN, α,α′-azodiisobutyronitrile; aq, aqueous; BnBr, benzyl bromide; DCM, dichloromethane; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; FAc, fluoro-acetyl; HBS-E, HEPES/NaCl/EDTA buffer; HBS-EP, HEPES–NaCl–EDTA–P20 buffer; ITC, isothermal titration calorimetry; K_D, dissociation constant; MS, mass spectrometry; Neu5Ac, *N*-acetylneuraminic acid; NgR, Nogo receptor; NMR, nuclear magnetic resonance; PDC, pyridinium dichromate; PPTS, pyridinium *p*-toluenesulfonate; RP, reversed phase; SPR, surface plasmon resonance; STD-NMR, saturation transfer difference nuclear magnetic resonance spectroscopy; THF, tetrahydrofuran.

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doi:10.1016/j.carres.2010.03.010

Please cite this article in press as: Mesch, S.; et al. *Carbohydr. Res.* (2010), doi:10.1016/j.carres.2010.03.010

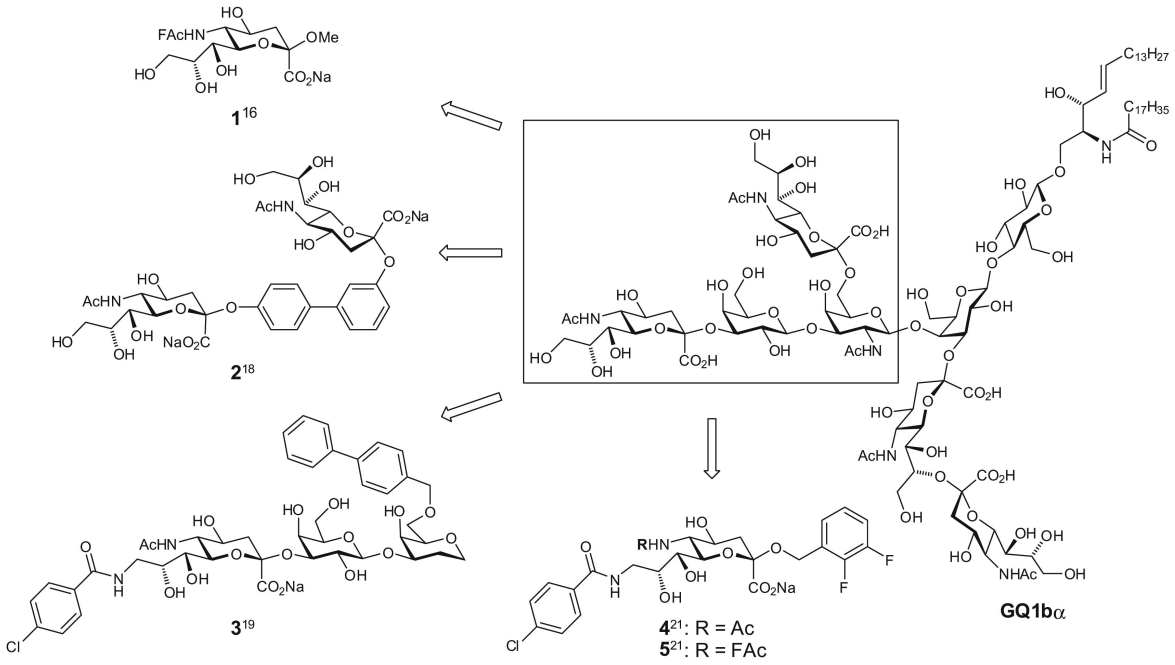

Figure 1. MAG antagonists **1**[16], **2**[18], **3**[19] and **4**, **5**[21] derived from the tetrasaccharide core structure (highlighted in box) of GQ1bα.

Table 1
Carbohydrate–protein interactions: thermodynamic and kinetic binding parameters

Protein	Ligand	K_D (μM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	$t_{1/2}$ (s)
P-Selectin[24]	PSGL-1	0.3	$4{\cdot}10^6$	1.4	0.5
E-Selectin[25]	ESL-1	62	$4{\cdot}10^4$	3.0	0.2
GSLA-2 mAb[26]	Sialyl Lewis[a]	4.3	$1.1{\cdot}10^5$	0.48	1.5
MAG[19]	Neu5Ac derivative **3**[19]	2.8	$3.5{\cdot}10^5$	0.8	0.9

of thermodynamic and kinetic parameters for carbohydrate–protein interactions are summarized in Table 1.

For medical applications, an improved $t_{1/2}$ of the drug–protein complex is beneficial, because the therapeutic effect can be reached with a lower dose. Zanamivir is one of the prominent examples, where a carbohydrate-based lead was optimized to yield a drug with a dramatically improved kinetic behavior, showing a half-life of 33 min of its complex with the B/Memphis/3/89 (H3N2) influenza virus.[27] In this communication, we present various MAG antagonists modified at the 4- and 5-position with the aim to modulate their kinetic properties. In general, lead optimization is often achieved by additional lipophilic contacts and thereby improving the binding entropy. As a result of the increased lipophilicity, the dissociation half-life ($t_{1/2}$) of the drug–target complex is extended.[28,29] The starting point for our investigation was MAG antagonist **5**,[21] a result of an extended optimization program focusing exclusively on the improvement of its thermodynamic binding properties.[17–19,21]

2. Results and discussion

Recently, we reported the synthesis and biological evaluation of a series of MAG antagonists with affinities in the low micromolar range.[21] Furthermore, pharmacokinetic parameters such as stability and membrane penetration indicated that the antagonists **4** and **5** (Fig. 1) fulfill the basic requirements for lead compounds. As halogenated acetates at the 5-position led to a drastic improvement of the binding affinity,[16,21] we investigated the impact of this position on the thermodynamic properties and also examined its influence on the dissociation half-life time. Molecular modeling studies with a homology model of MAG[30] suggested that the hydroxy group in the 4-position is not directly involved in the binding process[19] and therefore provides a possibility for derivatization. Because additional hydrophobic contacts based on the 4-position and an inverted configuration at C-4 are expected to alter the thermodynamic and kinetic behavior, we synthesized a small library of antagonists and analyzed their binding properties by surface plasmon resonance.

2.1. A MAG antagonist modified in the 5-position of the Neu5Ac scaffold

With isothermal titration calorimetry, we determined the thermodynamic parameters ΔH, ΔS, and ΔG[31] of antagonist **5** interacting with a recombinant protein consisting of the three N-terminal domains of MAG and the Fc part of human IgG ($MAG_{d1\text{-}3}$-Fc).[32] For the ITC experiment, a solution of **5** (500 μM, HBS-E buffer) was injected into a solution of $MAG_{d1\text{-}3}$-Fc (48.35 μM, HBS-E buffer) at 25 °C (Fig. 2).

The experimental data were fitted to a theoretical titration curve (one site binding model) using *Origin version 7* software (MicroCal) and the thermodynamic parameters calculated according to the equation shown in Table 2. The ITC experiment confirmed the high potency of **5**, having a K_D in the nanomolar

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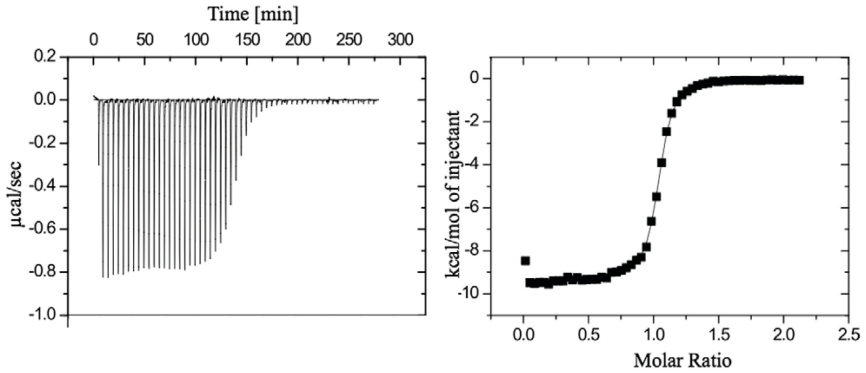


Figure 2. Enthalpogram (left) and corresponding fit (right) of the titration of MAG_{d1-3}-Fc with antagonist **5**. For the fit, the first injection was not taken into account.

Table 2
Thermodynamic parameters of antagonist **5**

Ligand	*N*	K_D (nM)	ΔG (kJ/mol)*	ΔH (kJ/mol)*	$T\Delta S$ (kJ/mol)*
5	1.03	142	−39.1	−39.2	−0.14

ΔH, ΔS, and ΔG were calculated according to the equation $\Delta G = \Delta H - T\Delta S = RT\ln K_A = -RT\ln K_D$; *N* represents the stoichiometry, * values' accuracy ±5%.

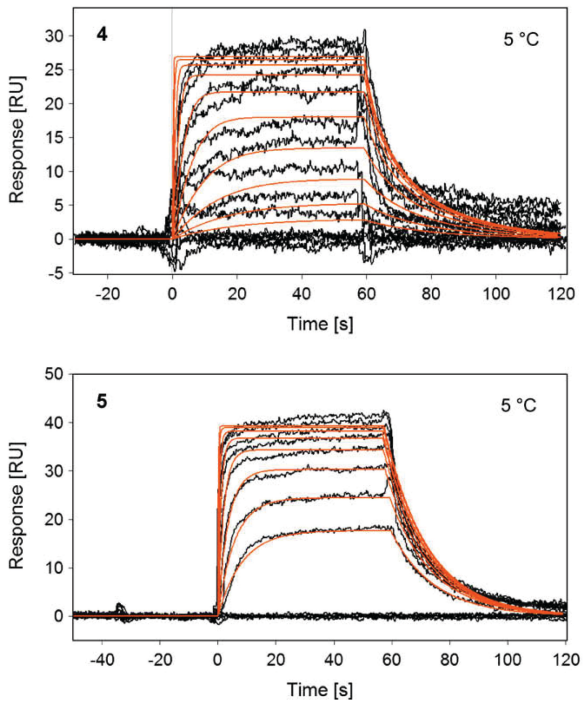
range.[21] Interestingly, the interaction is exclusively enthalpy driven.[33,34]

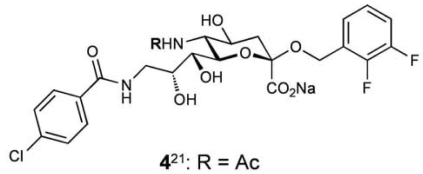
In a next step, the kinetic binding properties of **5** were determined by surface plasmon resonance (SPR) (Fig. 3). Because the off-rate at 25 °C turned out to be very fast, the Biacore experiment was repeated at lower temperature. At 5 °C, a clear slowdown of k_{off} for **5** (R: FAc) compared to that for **4** (R: Ac) was observed.

2.2. MAG antagonists modified in the 4-position of the Neu5Ac scaffold

As an increased lipophilicity often leads to prolonged residence times,[35] hydrophobic substituents were introduced in the 4-position. Furthermore, we planned to investigate the influence of the configuration at this position on the kinetic binding behavior.

Starting from the Neu5Ac donor **6**,[36] compound **8** was obtained according to published procedures (Scheme 1).[21] After deacetylation under Zemplén conditions (→**9**), the acetonide[37] **10** was formed with the two hydroxy groups in the 7- and 8-position, permitting selective modifications of the 4-position. To obtain the 4-deoxy compound **14**, the 4-hydroxy group in **10** was transformed into the corresponding thiocarbonate **12**, a precursor for a Barton deoxygenation using tributyltin hydride.[38] Cleavage of the aceto-





4[21]: R = Ac
5[21]: R = FAc

Compound	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]	$t_{1/2}$ [s]
4 5 °C	$9.4 \cdot 10^4$	0.153	4.5
5 5 °C	$2.75 \cdot 10^5$	0.07	9.9
5 25 °C	$2.8 \cdot 10^5$	0.154	4.5

Figure 3. Kinetic fits of compounds **4** and **5** at 5 °C. In the case of **5**, an increase of the dissociation half-life ($t_{1/2}$) by a factor of 2 was observed. For the fitting of the sensorgrams Scrubber 2.0c was applied.

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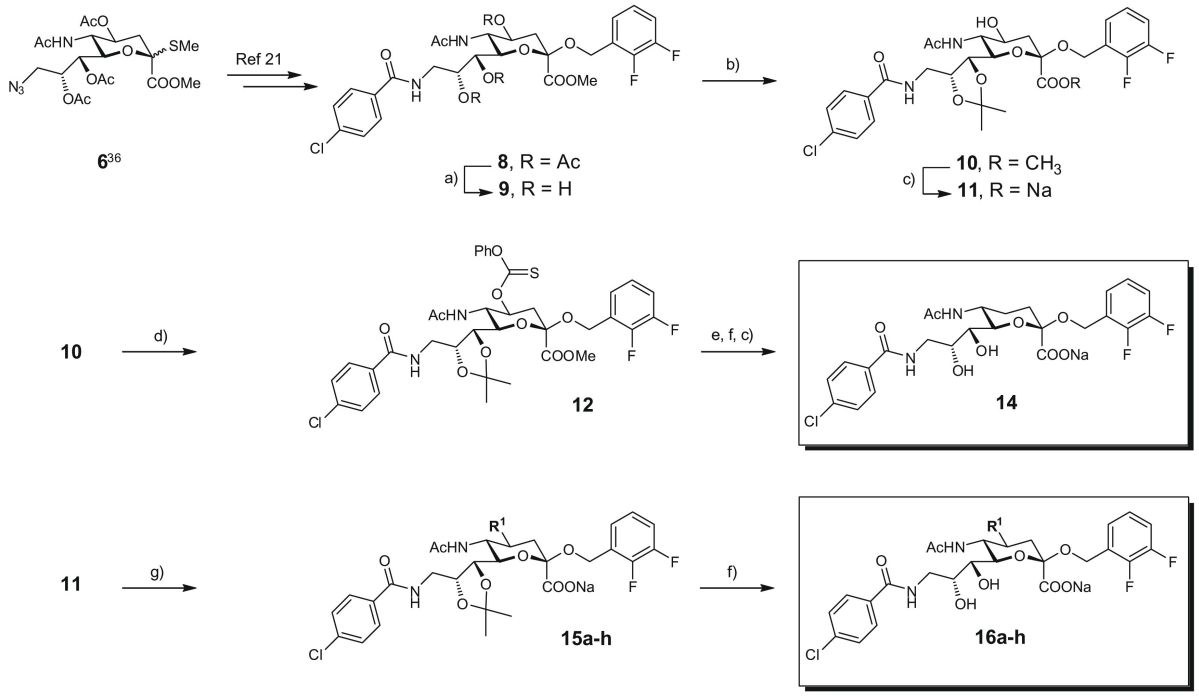


Scheme 1. Reagents and conditions: (a) NaOMe, MeOH (61%); (b) $MeO_2C(CH_3)_2$, PPTS, MeCN (60%); (c) 10% NaOH (aq), MeOH (42%); (d) $C_7H_5ClO_2S$, DCM, pyridine (83%); (e) *n*-Bu_3SnH, AIBN, toluene, 100 °C (→**13**, 20%); (f) 80% AcOH (aq), 60 °C (→**16a–h**, 10–80%); (g) (i) $(R^1O)_2C{=}O$, DMAP, pyridine (→**15a**, 80%, **15b**, 61%); or (ii) BnBr, KOH (aq, 50%), 18-crown-6, DCM, 60 °C (→**15c**, 60%); or (iii) R^1NCO, DMAP, pyridine (→**15d–h**, 20–37%).

nide under acidic conditions followed by hydrolysis of the methyl ester yielded test compound **14** in excellent overall yield.

Starting from **11**, the hydroxy group in the 4-position was either acylated with the corresponding anhydrides (→**15a**,**b**), the corresponding isocyanides (→**15d–h**), or reacted with benzyl bromide under phase-transfer catalysis conditions (→**15c**). Finally, the acetonide was cleaved under acidic conditions to yield the test compounds **16a–h** (Scheme 1, Table 3).

The 4-disubstituted antagonist **19** was obtained in a two-step procedure (Scheme 2). Oxidation of **10** with pyridinium dichromate under acidic conditions[39] yielded **17**; however, due to the instability of the acetonide only in moderate 30% yield. Various other conditions, for example, using molecular sieves instead of acetic anhydride[40] did not lead to notably improved yields. Then, **17** was reacted with the tetramethylzirconium complex[41] followed by the cleavage of the acetonide to yield **18** with an acceptable stereoselectivity (11% of the *S*-stereoisomer was formed). The zirconium complex was chosen in order to avoid undesired side reactions (e.g., enolization) as reported earlier by Hartmann et al.[42] Final deprotection gave compound **19**.

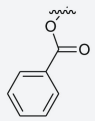
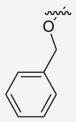
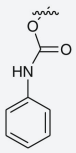
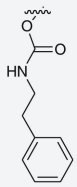
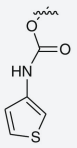
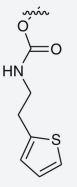
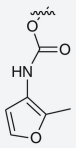
Test compound **22** was obtained by reduction of **17** with $BH_3{\cdot}NH_3$, yielding the 4-hydroxy compound **20** [(*R*)-stereoisomer] with the inverted configuration at C-4 compared to Neu5Ac.[43] Finally, removal of the acetonide and hydrolysis of the methyl ester gave compound **22**.

2.3. Biological evaluation and kinetic studies

First, the affinity of the test compounds **14**, **16a–h**, **19**, and **22** toward MAG was determined by a surface plasmon resonance based biosensor (Biacore) experiment.[21] Fc-$MAG_{d1\text{-}3}$-Fc[32] was immobilized on a dextran chip containing a surface of covalently bound protein A. A reference cell providing only protein A was used to compensate unspecific binding to the matrix.

Dilution series of the compounds were prepared either in pure HEPES-buffer or in buffer containing 3% DMSO and passed over the flow cells. As reported earlier,[21] negative sensorgrams were obtained (after subtraction of the reference cell). After their mirroring, they could be fitted to a one-to-one binding model using Scrubber 2.0c (Table 4). The kinetic parameters k_{on} and k_{off} were obtained by applying a global fit (Scrubber 2.0c).

Table 3
Substituents R^1 in antagonist **16**

16a	16b	16c	16d	16e	16f	16g	16h
							

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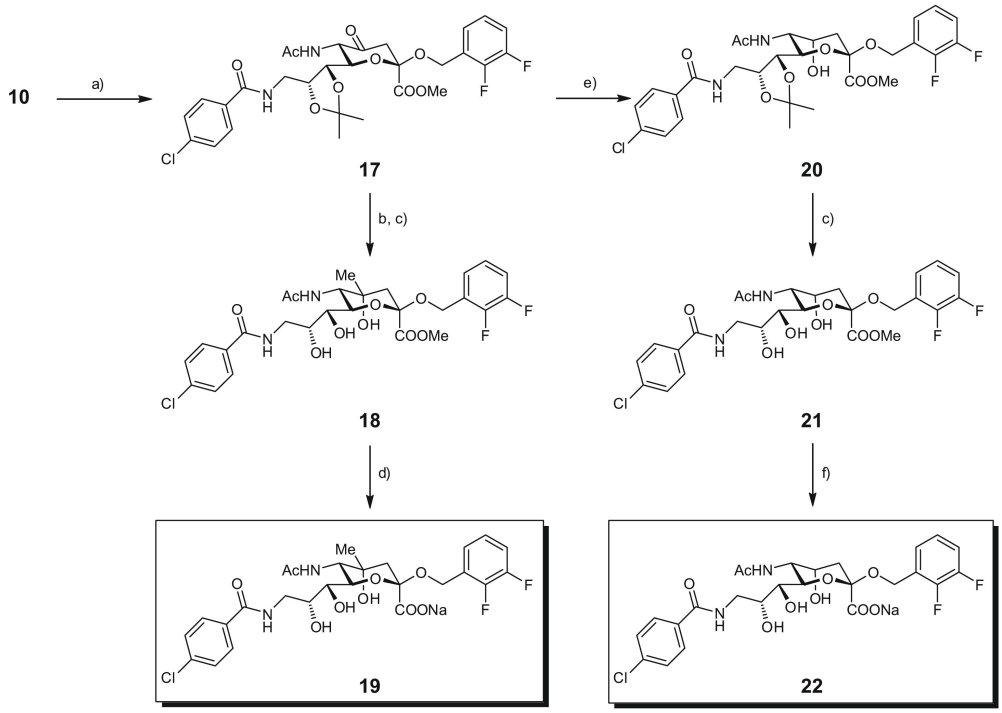
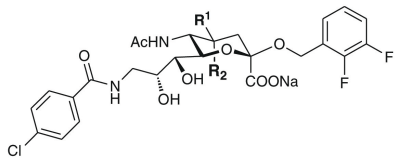
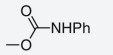
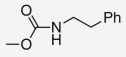
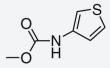
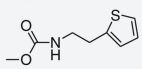
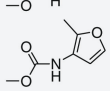


Scheme 2. Reagents and conditions: (a) PDC, Ac_2O, DCM, rt (30%); (b) Me_4Zr, THF, −78 °C, 50% NH_4Cl (aq); (c) 80% AcOH (aq), 60 °C (→**18**, 32 + 11% (*S*)-stereoisomer, two steps; →**21**, 90%); (d) LiOH (aq), THF (34%); (e) $BH_3{\cdot}NH_3$, MeOH, 0 °C (36%); (f) 10% NaOH (aq) (34%).

Table 4
Affinities and kinetic parameters of Neu5Ac derivatives modified in the 4-position



Compd	R^1	R^2	K_D (μM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	$t_{1/2}$ (s)
4[21]	OH	H	2.0	1.5×10^5	0.54	1.2
14	H	H	11.4	2.8×10^4	0.28	2.5
16a	OAc	H	11.5	4.6×10^4	0.52	1.3
16b	OBz	H	26.0	1.6×10^4	0.41	1.7
16c	OBn	H	2.1	1.6×10^4	0.33	2.1
16d		H	257	2.7×10^4	7.00	0.1
16e		H	114	5.3×10^6	>100	<0.01
16f		H	15.6	n.d.	n.d.	n.d.
16g		H	n.b.	n.d.	n.d.	n.d.
16h		H	49.4	2.2×10^4	1.10	0.6
19	Me	OH	30.0	2.4×10^{-4}	0.53	1.3
22	H	OH	9.0	3.6×10^{-4}	0.33	2.1

n.d., not determined; n.b., not binding.

The deoxy compound **14** showed a decrease in affinity by a factor of six. The inversion of the configuration at the 4-position (→**22**) or the introduction of a methyl group in equatorial position (→**19**) led also to a drop in binding affinity (factor of 4 and 15, respectively). Originally, we assumed based on docking studies to a homology model of MAG[30] that the hydroxy group in the 4-position is not directed toward the protein and can therefore be modified. Our results, however, suggest that the equatorial 4-hydroxy contributes to binding, maybe by hydrogen bonding, as the change of the configuration at C-4 also leads to a decreased affinity. Furthermore, a steric clash of the methyl group in **19** could be responsible for a further reduction of affinity.

In case of antagonists **16a**,**b** and **16d**–**h**, a pronounced drop in the binding affinity or even a complete abolishment of binding was observed. In contrast, **16c** showed the same binding affinity as reference compound **4**. The loss in potency for **16a**,**b** and **16d**–**h** is probably the consequence of the rigid geometry of ester and carbamate substituents, leading to a steric clash with the protein, whereas the benzyl ether in **16c** seems to adapt a favorable spatial arrangement compensating the impact of the 4-hydroxy to binding affinity.

Beside the decreased binding affinity, no substantial improvement of the kinetic properties could be achieved. All compounds showed fast dissociation rate constants, leading to residence times in the range of seconds (Table 4). Despite the increased lipophilicity, even **16c** does not show an extended dissociation half-life.

3. Conclusion

A small library of MAG antagonists modified in the 4-position of the Neu5Ac scaffold was synthesized with the goal to improve the half-life of the antagonist-MAG complex. Although all modifications in the 4-position were not successful, the investigation of

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modifications in the 5-position led to a reduction of the dissociation rate constant k_{off}, although only by a factor of 2. In conclusion, the prolongation of the residence time remains a challenge and will be a critical issue in further studies on the kinetic properties of glycomimetics.

4. Experimental

4.1. General methods

NMR spectra were recorded on a Bruker Avance DMX-500 (500 MHz) spectrometer. Assignment of ^{1}H and ^{13}C NMR spectra was achieved using 2D methods (COSY, HSQC, TOCSY, ROESY, and NOESY). Chemical shifts are expressed in ppm using residual $CHCl_3$, CHD_2OD, CHD_2CN, and HDO as references. Optical rotations were measured using Perkin–Elmer Polarimeters 241 and 341. MS analyses were carried out using a Waters Micromass ZQ Detector system. The spectra were recorded in positive or negative ESI mode. Reactions were monitored by TLC using glass plates coated with Silica Gel 60 F_{254} (Merck) and visualized by using UV light and/or by charring with a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aq 10% H_2SO_4). Column chromatography was performed on silica gel (Fluka, 40–60 mesh). MeOH was dried by heating at reflux with sodium methoxide and distilled immediately before use. Pyridine was freshly distilled under argon over CaH_2. Dichloromethane (DCM), dichloroethane (DCE), acetonitrile (MeCN), toluene, and benzene were dried by filtration over Al_2O_3 (Fluka, type 5016 A basic). Molecular sieves (3 or 4 Å) were activated in vacuo at 500 °C for 2 h immediately before use. Compounds **6**–**8** were prepared according to a published procedure.[21,36]

4.2. Synthesis and characterization of compounds 9–22

4.2.1. Methyl [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (9)

Compound **8**[21] (217 mg, 42.0 mmol) was dissolved in dry MeOH (8.0 mL) and treated with methanolic NaOMe (1 M, 1.0 mL) for 2 h. The reaction mixture was neutralized with Amberlyst 15, filtered over a pad of Celite, and the Celite was washed thoroughly with MeOH. The solvent was evaporated under reduced pressure and the crude product was purified by chromatography on silica gel (1% gradient of MeOH in DCM) to yield **9** (90.0 mg, 61%) as a white foam. ^{1}H NMR (500 MHz, CD_3OD) δ 1.80 (t, J = 12.3 Hz, 1H, H-3a), 1.97 (s, 3H, NHAc), 2.72 (dd, J = 4.5, 12.8 Hz, 1H, H-3b), 3.45 (d, J = 8.8 Hz, 1H, H-7), 3.55 (dd, J = 7.3, 13.8 Hz, 1H, H-9a), 3.61–3.74 (m, 2H, H-4, H-6), 3.75–3.90 (m, 5H, OMe, H-5, H-9), 4.04 (td, J = 3.2, 8.8 Hz, 1H, H-8), 4.68, 4.86 (A, B of AB, J = 12.1 Hz, 2H, CH_2Ar), 7.06–7.24 (m, 3H, CH_{ar}), 7.46, 7.82 (AA′, BB′ of AA′BB′, J = 8.4 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.7 (NHAc), 41.6 (C-3), 45.1 (C-9), 53.4 (OMe), 53.8 (C-5), 68.5 (C-4), 70.8 (C-8), 72.1 (C-7), 74.9 (C-6), 117.8, 125.4, 126.4, 128.0, 129.7, 130.1, 131.3, 133.0, 134.4, 138 (12C, C-Ar), 169.6, 170.6, 175.0 (3CO). ESI-MS calcd for $C_{26}H_{29}ClF_2N_2O_9$ $[M+Na]^+$: 609.14; found m/z 609.19.

4.2.2. Methyl [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (10)

Compound **9** (20.0 mg, 30 μmol) was dissolved in dry MeCN (250 μL) and 2,2-dimethoxypropane (17 μL, 0.12 mmol) was added. After cooling to 0 °C PPTS (3.0 mg, 10 μmol) was added. The reaction mixture was stirred at rt overnight. After completion of the reaction, IRA-93 was added and stirring was continued for 30 min. After filtration, the solvent was evaporated and the pure product **10** (13 mg, 60%) was obtained by chromatography on silica gel (1% gradient of MeOH in DCM). ^{1}H NMR (500 MHz, CD_3OD): δ 1.33, 1.51 (2s, 6H, 2 C(CH_3)$_2$), 1.75 (t, J = 12.4 Hz, 1H, H-3a), 1.99 (s, 3H, NHAc), 2.67 (dd, J = 4.3, 12.6 Hz, 1H, H-3b), 3.56–3.65 (m, 1H, H-4), 3.83 (s, 3H, OMe), 3.86–4.00 (m, 3H, H-5, H-6, H-9a), 4.03 (dd, J = 3.1, 14.1 Hz, 1H, H-9b), 4.26 (d, J = 6.8 Hz, 1H, H-7), 4.48 (ddd, J = 3.3, 7.1, 8.7 Hz, 1H, H-8), 4.56–4.69 (m, 1H, CH_2Ar), 4.96 (B of AB, J = 11.6 Hz, 1H, CH_2Ar), 7.09–7.27 (m, 4H, NH, CH_{ar}), 7.46, 7.83 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 23.0 (NHAc), 25.8, 27.1 (C(CH_3)$_2$), 41.6, 41.8 (2C, C-3, C-9), 53.2 (OMe), 54.3 (C-5), 61.0 (CH_2Ar), 68.4 (C-4), 74.3 (C-6), 75.8 (C-7), 77.7 (C-8), 100.5 (C-2), 110.2 (C(CH_3)$_2$), 117.9, 118.0, 125.4, 126.7, 129.7, 130.1, 130.2, 134.3, 138.7 (12C, C-Ar), 169.3, 170.4, 173.9 (3CO). ESI-MS calcd for $C_{29}H_{33}ClF_2N_2O_9$ $[M+Na]^+$: 649.18; found m/z 649.15.

4.2.3. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (11)

Compound **10** (20.0 mg, 30 μmol) was dissolved in MeOH (2 mL) and 10% aq NaOH (0.1 mL) was added. After 2 h, the solution was neutralized with 7% aq HCl. Then the solvent was evaporated and the pure product **11** (13 mg, 42%) was obtained by chromatography on RP-8 (10% of MeOH in water). $[\alpha]_D^{20}$ −33.4 (c 0.37, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 1.27, 1.41 (2s, 6H, 2 (CH_3)$_2$), 1.65 (t, J = 12.0 Hz, 1H, H-3a), 1.92 (s, 3H, NHAc), 2.75 (dd, J = 4.3, 12.0 Hz, 1H, H-3b), 3.57 (ddd, J = 4.3, 10.3, 12.0 Hz, 1H, H-4), 3.82–3.99 (m, 3H, H-5, H-9a, H-9b), 4.11 (d, J = 10.3 Hz, 1H, H-6), 4.18 (d, J = 6.8 Hz, 1H, H-7), 4.35 (dd, J = 7.1, 12.5 Hz, 1H, H-8), 4.61, 4.89 (A, B of AB, J = 10.6 Hz, 2H, CH_2Ar), 7.00–7.16 (m, 2H, CH_{ar}), 7.30 (t, J = 6.8 Hz, 1H, CH_{ar}), 7.40, 7.91 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.9 (NHAc), 25.4, 27.4 (C(CH_3)$_2$), 41.9, 42.1 (2C, C-3, C-9), 54.7 (C-5), 60.3 (CH_2Ar), 69.75 (C-4), 73.1 (C-6), 75.8 (C-7), 76.5 (C-8), 102.8 (C-2), 109.3 (C(CH_3)$_2$), 117.0, 117.1, 125.2, 126.5, 129.6, 130.6, 134.1, 138.6 (12C, C-Ar), 169.5, 173.9, 174.4 (3CO). ESI-MS calcd for $C_{28}H_{30}ClF_2N_2NaO_9$ $[M+H]^+$: 634.15; found m/z 635.16.

4.2.4. Methyl [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-4-*O*-(phenoxycarbonothioyl)-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (12)

Compound **10** (96 mg, 0.15 mmol) was dissolved in dry pyridine/DCM (2/1; 1.5 mL). After cooling to 0 °C phenyl chlorothioformate (162 μL, 1.20 mmol) was added dropwise. Stirring was continued for 2 h at rt and then MeOH (2 mL) was added. After evaporation of the solvents, the residue was dissolved in DCM (5 mL) and washed with 0.5 M aq $CuSO_4$ (1 mL) and water (1 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by chromatography on silica gel (1% gradient of MeOH in DCM) to yield **12** (14 mg, 83%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.37, 1.54 (2s, 6H, C(CH_3)$_2$), 1.97–2.00 (m, 4H, NHAc, H-3a), 3.01 (dd, J = 4.6, 12.3 Hz, 1H, H-3b), 3.87 (s, 3H, OMe), 3.96–4.02 (m, 1H, H-9a), 4.05 (dd, J = 3.2, 14.2 Hz, 1H, H-9b), 4.15 (d, J = 10.3 Hz, 1H, H-6), 4.27–4.37 (m, 2H, H-5, H-7), 4.53 (td, J = 3.4, 8.6 Hz, 1H, H-8), 4.66, 5.02 (A, B of AB, J = 11.5 Hz, 2H, CH_2Ar), 5.59 (td, J = 4.6, 12.0 Hz, 1H, H-4), 7.07 (d, J = 8.3 Hz, 2H, CH_{ar}), 7.13–7.18 (m, 1H, CH_{ar}), 7.21–7.27 (m, 2H, CH_{ar}), 7.30 (t, J = 7.4 Hz, 1H, CH_{ar}), 7.43 (t, J = 7.9 Hz, 2H, CH_{ar}), 7.48, 7.84 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 23.0 (NHAc), 25.8, 27.0 (C(CH_3)$_2$), 37.9 (C-3), 41.7 (C-9), 51.3 (C-5), 53.4 (OMe), 61.3 (CH_2Ar), 73.9 (C-6), 75.5 (C-7), 77.7 (C-8), 80.1 (C-4), 100.1 (C-2), 110.4 (C(CH_3)$_2$), 118.0, 122.1, 122.9, 125.5, 126.8, 126.9, 127.3, 127.7, 129.7, 130.1, 130.6, 134.2, 138.8, 152.6, 154.6, 154.9 (18C, C-Ar), 169.3,

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169.8, 173.5 (3CO), 196.0 (CS). ESI-MS calcd for $C_{36}H_{37}ClF_2N_2O_{10}S$ $[M+Na]^+$: 785.17; found *m*/*z* 785.27.

4.2.5. Methyl [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,4,5,9-tetradeoxy-7,8-*O*-isopropylidene-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (13)

Compound **12** (96 mg, 0.13 mmol) was dissolved in dry toluene (4 mL). After the sequential addition of freshly distilled *n*-Bu_3SnH (340 μL, 1.3 mmol) and AIBN (29 mg, 0.18 mmol), the reaction mixture was stirred at 100 °C for 1 h. After evaporation of the solvent, the crude product was purified by chromatography on silica gel (1% gradient MeOH in DCM) to yield **13** (15.3 mg, 20%) as a white foam. ^{1}H NMR (500 MHz, CD_3OD): δ 1.33 (s, 3H, CH_3), 1.35–1.42 (m, 1H, H-4a), 1.51 (s, 3H, CH_3), 1.81 (td, J = 4.0, 13.8 Hz, 1H, H-3a), 1.94 (s, 3H, NHAc), 2.00–2.07 (m, 1H, H-4b), 2.37 (dt, J = 3.4, 13.1 Hz, 1H, H-3b), 3.81 (s, 3H, OMe), 3.92–4.02 (m, 2H, H-9a, H-9b), 4.02–4.08 (m, 2H, H-5, H-6), 4.26 (d, J = 7.6 Hz, 1H, H-7), 4.48 (ddd, J = 3.3, 7.0, 8.6 Hz, 1H, H-8), 4.56, 4.96 (A, B of AB, J = 11.6 Hz, 2H, CH_2Ar), 7.10–7.20 (m, 3H, CH_{ar}), 7.46, 7.83 (AA′, BB′ of AA′BB′, J = 8.7 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.8 (NHAc), 25.8, 27.1 (C(CH_3)$_2$), 27.5 (C-4), 32.8 (C-3), 41.7 (C-9), 46.2 (C-5), 53.0 (OMe), 61.0 (CH_2Ar), 75.9, 76.4, 77.7 (C-6, C-7, C-8), 100.9 (C-2), 110.2 (C(CH_3)$_2$), 117.8, 125.4, 126.7, 129.7, 130.1, 134.3, 138.7 (12C, C-Ar), 169.3, 170.8, 172.9 (3CO). ESI-MS calcd for $C_{29}H_{33}ClF_2N_2O_8$ $[M+Na]^+$: 633.18; found *m*/*z* 633.12.

4.2.6. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,4,5,9-tetradeoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (14)

Compound **13** (15.0 mg, 20 μmol) was dissolved in 80% aq AcOH (2.5 mL) and heated for 3 h at 60 °C. After TLC showed completion of the reaction, the reaction mixture was cooled to rt. The pH was set to 10 by addition of 10% aq NaOH. Stirring was continued for 4 h and then the reaction mixture was neutralized with 7% aq HCl. After evaporation of the solvents, the crude product was purified by chromatography on RP-8 (5% gradient of MeOH in water) to yield **14** (4.5 mg, 34%) as a white solid. $[\alpha]_D^{20}$ −29.7 (*c* 0.13, MeOH); ^{1}H NMR (500 MHz, D_2O): δ 1.47 (q, J = 11.2 Hz, 1H, H-4a), 1.68 (td, J = 3.7, 13.3 Hz, 1H, H-3a), 1.94 (s, 3H, NHAc), 2.00–2.09 (m, 1H, H-4b), 2.35–2.47 (m, 1H, H-3b), 3.40–3.58 (m, 2H, H-7, H-9a), 3.70–3.81 (m, 3H, H-6, H-8, H-9b), 3.87 (td, J = 4.0, 11.1 Hz, 1H, H-5), 4.61, 4.79 (A, B of AB, J = 11.8 Hz, 2H, CH_2Ar), 7.00–7.27 (m, 3H, CH_{ar}), 7.53, 7.75 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, D_2O): δ 21.8 (C-4), 26.9 (NHAc), 31.7 (C-3), 44.5 (C-9), 51.7 (C-5), 60.5 (CH_2Ar), 68.2, 70.1 (C-7, C-8), 75.8 (C-6), 99.3 (C-2), 117.0, 124.3, 125.8, 126.5, 128.7, 128.8, 132.1, 137.5 (12C, C-Ar), 174.2, 186.3 (3C, 3CO). HRMS calcd for $C_{25}H_{26}ClF_2N_2NaO_{11}$ $[M]^+$: 601.1141; found *m*/*z* 601.1154.

4.2.7. Sodium [(2,3-difluorobenzyl) 5-acetamido-4-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (15a)

Compound **11** (13.2 mg, 20 μmol) was dissolved in dry pyridine (0.5 mL). After cooling to 0 °C, acetic anhydride (0.25 mL, 2.64 mmol) and DMAP (1.0 mg, 8.0 μmol) were added. The reaction mixture was allowed to reach rt and stirring was continued for 24 h. After completion of the reaction, the solvent was removed by co-evaporation with toluene. Then DCM (5 mL) was added and the organic layer was washed with 0.5 M aq $CuSO_4$ (2 mL), satd aq $NaHCO_3$ (3 × 2 mL), and H_2O (2 mL). Finally, the crude product was purified by chromatography on silica gel (10% gradient MeOH in DCM) to yield **15a** (11.2 mg, 80%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.29, 1.43 (2s, 6H, C(CH_3)$_2$), 1.70 (t, J = 11.5 Hz, 1H, H-3a), 1.86 (s, 3H, NHAc), 1.93 (s, 3H, OAc), 2.75 (dd, J = 4.5, 11.5 Hz, 1H, H-3b), 3.86–4.07 (m, 2H, H-5, H-9a), 4.15 (dd, J = 4.0, 14.5 Hz, 1H, H-9b), 4.20–4.28 (m, 2H, H-6, H-7), 4.39–4.46 (m, 1H, H-8), 4.63, 4.93 (A, B of AB, J = 11.9 Hz, 2H, CH_2Ar), 5.05–5.17 (m, 1H, H-4), 7.00–7.14 (m, 2H, CH_{ar}), 7.16–7.27 (m, 1H, CH_{ar}), 7.37, 7.84 (AA′, BB′ of AA′BB′, J = 7.9 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 20.9 (OAc), 22.9 (NHAc), 25.8, 27.2 (C(CH_3)$_2$), 39.2, 40.2 (C-3, C-9), 52.4 (C-5), 60.4 (CH_2Ar), 71.5, 72.7, 75.8, 76.9 (C-4, C-6, C-7, C-8), 101.3 (C-2), 110.1 (C(CH_3)$_2$), 117.2, 117.3, 125.3, 126.4, 129.6, 130.5, 138.9, 140.1 (12C, C-Ar), 170.6, 172.3, 173.5, 177.9 (4CO). ESI-MS calcd for $C_{30}H_{32}ClF_2N_2NaO_{10}$ $[M+H]^+$: 677.16; found *m*/*z* 677.21.

4.2.8. Sodium [(2,3-difluorobenzyl) 5-acetamido-4-*O*-benzoyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (15b)

Compound **11** (10.0 mg, 20 μmol) was dissolved in dry pyridine (0.5 mL) at rt. After cooling to 0 °C, benzoic anhydride (100 mg, 0.44 mmol) and DMAP (1.0 mg, 8.0 μmol) were added. The reaction mixture was allowed to reach rt and stirring was continued for 24 h. After completion of the reaction, the solvent was removed by co-evaporation with toluene. Then DCM (5 mL) was added and the organic layer was washed with 0.5 M aq $CuSO_4$ (2 mL), satd aq $NaHCO_3$ (3 × 2 mL), and H_2O (2 mL). Finally, the crude product was purified by chromatography on silica gel (10% gradient of MeOH in DCM) to yield **15b** (7.1 mg, 61%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.31, 1.47 (2s, 6H, C(CH_3)$_2$), 1.76 (s, 3H, NHAc), 1.82–1.96 (m, 1H, H-3a), 2.93 (dd, J = 4.2, 11.8 Hz, 1H, H-3b), 3.97 (dd, J = 6.0, 13.4 Hz, 1H, H-9a), 4.10–4.20 (m, 1H, H-9b), 4.25 (d, J = 6.5 Hz, 1H, H-7), 4.27–4.37 (m, 2H, H-5, H-6), 4.46 (dd, J = 6.1, 12.4 Hz, 1H, H-8), 4.67, 4.98 (A, B of AB, J = 11.7 Hz, 2H, CH_2Ar), 5.23–5.40 (m, 1H, H-4), 6.98–7.15 (m, 2H, CH_{ar}), 7.27 (t, J = 6.5 Hz, 1H, CH_{ar}), 7.42 (d, J = 7.9 Hz, 2H, CH_{ar}), 7.48 (t, J = 7.4 Hz, 2H, CH_{ar}), 7.55 (t, J = 7.5 Hz, 1H, CH_{ar}), 7.88 (d, J = 8.3 Hz, 2H, CH_{ar}), 7.92 (d, J = 7.5 Hz, 2H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.8 (NHAc), 25.7, 27.3 (C(CH_3)$_2$), 30.8 (C-3), 39.3 (C-9), 42.3 (C-5), 52.0 (CH_2Ar), 72.6, 72.9, 75.9, 76.8 (C-4, C-6, C-7, C-8), 110.0 (C-2), 111.4 (C(CH_3)$_2$), 117.3, 129.1, 129.5, 129.6, 130.5, 130.6, 130.7, 131.2, 133.0, 134.3, 135.1 (18C, C-Ar), 172.0 (4C, 4CO). ESI-MS calcd for $C_{35}H_{34}ClF_2N_2NaO_{10}$ $[M+Na]^+$: 739.18; found *m*/*z* 739.27.

4.2.9. Sodium [(2,3-difluorobenzyl) 5-acetamido-4-*O*-benzyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (15c)

Compound **11** (10 mg, 20 μmol) was dissolved in DCM/50% aq KOH (1/3, 0.5/1.5 mL). After adding 18-crown-6 (1.0 mg) and benzyl bromide (60 μL, 0.5 mmol), the reaction mixture was heated to 60 °C and stirring was continued for 18 h. The solvents were removed under reduced pressure and the crude product was purified by chromatography on silica gel (10% gradient of MeOH in DCM) to yield **15c** (6.8 mg, 60%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.32, 1.47 (2s, 6H, C(CH_3)$_2$), 1.64–1.78 (m, 1H, H-3a), 1.94 (s, 3H, NHAc), 3.02 (dd, J = 4.0, 12.0 Hz, 1H, H-3b), 3.56–3.64 (m, 1H, H-4), 3.97 (dd, J = 6.1, 13.3 Hz, 1H, H-9a), 4.00–4.11 (m, 2H, H-5, H-9b), 4.22 (d, J = 10.5 Hz, 1H, H-6), 4.27 (d, J = 6.7 Hz, 1H, H-7), 4.44 (dd, J = 6.4, 12.8 Hz, 1H, H-8), 4.49 (A of AB, J = 12.0 Hz, 1H, CH_2Ar), 4.69 (A′, B′ of A′B′, J = 11.9 Hz, 2H, CH_2Ar), 4.99 (B of AB, J = 11.8 Hz, 1H, CH_2Ar), 7.08–7.19 (m, 3H, CH_{ar}), 7.22–7.28 (m, 5H, CH_{ar}), 7.46, 7.92 (AA′, BB′ of AA′BB′, J = 8.4 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 21.5 (NHAc), 24.1, 25.8 (C(CH_3)$_2$), 37.8 (C-3), 40.5 (C-9), 51.7 (C-5), 58.8 (CH_2Ar), 70.2 (C-6), 71.7 (CH_2Ar), 74.4 (C-4), 75.3, 75.4 (C-7, C-8), 100.7 (C-2), 108.1 (C(CH_3)$_2$), 115.6, 115.7, 123.8, 124.9, 127.0, 127.1, 127.8, 128.1, 128.9, 129.0, 132.6, 137.1, 138.6 (18C, C-Ar), 167.8, 171.9, 173.4 (3CO). ESI-MS calcd for $C_{35}H_{36}ClF_2N_2NaO_9$ $[M-H]^-$: 701.21; found *m*/*z* 701.52.

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4.2.10. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-4-*O*-phenylcarbamoyl-D-*glycero*-α-D-*galacto*-2-nonulopyranosid] onate (15d)

Compound **11** (30.0 mg, 50 μmol) was dissolved in dry pyridine (1.0 mL). Phenyl isocyanate (24 μL, 0.24 mmol) and DMAP (1.0 mg, 8.0 μmol) were added. The reaction mixture was stirred for 24 h and then water (0.1 mL) was added. After removal of the solvents under reduced pressure, the crude product was purified by chromatography on silica gel (10% gradient of MeOH in DM) to yield **15d** (10 mg, 29%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.25, 1.38 (2s, 6H, C(CH_3)$_2$), 1.85–1.92 (m, 4H, NHAc, H-3a), 2.76 (dd, J = 4.8, 13.0 Hz, 1H, H-3b), 3.50–3.64 (m, 1H, H-9a), 3.79–3.94 (m, 1H, H-9b), 4.10 (d, J = 10.4 Hz, 1H, H-7), 4.28–4.35 (m, 3H, H-5, H-6, H-8), 4.58, 4.70 (A, B of AB, J = 11.9 Hz, 2H, CH_2Ar), 5.21 (td, J = 4.7, 11.0 Hz, 1H, H-4), 6.96 (t, J = 7.3 Hz, 1H, CH_{ar}), 7.00–7.13 (m, 2H, CH_{ar}), 7.21 (dd, J = 7.7, 8.3 Hz, 2H, CH_{ar}), 7.23–7.28 (m, 1H, CH_{ar}), 7.31 (AA′ of AA′BB′, J = 8.6 Hz, 2H, CH_{ar}), 7.35 (d, J = 8.0 Hz, 2H, CH_{ar}), 7.84 (BB′ of AA′BB′, J = 8.6 Hz, 2H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.9 (NHAc), 25.5, 27.6 (C(CH_3)$_2$), 38.3 (C-3), 42.5 (C-9), 52.1 (C-5), 60.7 (CH_2Ar), 70.9, 71.6 (C-4, C-7), 76.1, 76.8 (C-6, C-8), 101.8 (C-2), 109.3 (C(CH_3)$_2$), 117.5, 119.7, 124.0, 125.6, 126.0, 128.6, 128.7, 129.4, 129.5, 129.8, 130.6, 134.0, 138.6, 140.1, 150.4, 150.5, 152.4 (18C, C-Ar), 155.1 (NC(O)O), 169.4, 173.7, 173.9 (3CO). ESI-MS calcd for $C_{35}H_{35}ClF_2N_3O_{10}$ [M−H]$^-$: 730.20; found m/z 730.33.

4.2.11. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-4-*O*-phenylethylcarbamoyl-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (15e)

Compound **11** (30.0 mg, 50 μmol) was dissolved in dry pyridine (1.5 mL). Phenylethyl isocyanate (50 μL, 0.42 mmol), DMAP (3.0 mg, 24.0 μmol), and NEt_3 (20 μL) were added. The reaction mixture was stirred at 60 °C for 24 h and then water (0.2 mL) was added. After removal of the solvents under reduced pressure, the crude product was purified by chromatography on silica gel (10% gradient of MeOH in DCM) to yield **15e** (7.3 mg, 20%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.30, 1.35 (2s, 6H, (CH_3)$_2$), 1.79 (t, J = 12.3 Hz, 1H, H-3a), 1.88 (s, 3H, NHAc), 2.61–2.78 (m, 3H, H-3b, CH_2), 3.18–3.26 (m, 2H, CH_2), 3.53–3.70 (m, 1H, H-9a), 3.87 (d, J = 12.1 Hz, 1H, H-9b), 4.07 (d, J = 10.6 Hz, 1H, H-7), 4.18–4.26 (m, 2H, H-5, H-6), 4.27–4.36 (m, 1H, H-8), 4.56, 4.69 (A, B of AB, J = 11.6 Hz, 2H, CH_2Ar), 5.08 (td, J = 4.8, 11.0 Hz, 1H, H-4), 7.01–7.09 (m, 2H, CH_{ar}), 7.14 (d, J = 7.5 Hz, 4H, CH_{ar}), 7.19–7.27 (m, 2H, CH_{ar}), 7.31, 7.83 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}); ^{13}C NMR (CD_3OD) δ 23.1 (NHAc), 25.8, 27.7 (C(CH_3)$_2$), 36.8 (CH_2), 38.4 (C-3), 42.6 (C-9), 43.4 (CH_2), 52.1 (C-5), 60.7 (CH_2Ar), 70.9 (C-7), 71.5 (C-4), 75.7 (C-6), 76.8 (C-8), 101.7 (C-2), 109.2 (C(CH_3)$_2$), 117.4, 125.5, 125.2, 126.0, 127.4, 128.7, 129.5, 129.9, 130.0, 130.4, 130.6, 134.1, 140.5, 150.5 (18C, C-Ar), 158.2 (COONH), 169.4, 173.5, 173.6 (3CO). ESI-MS calcd for $C_{37}H_{39}ClF_2N_3O_{10}$ [M−H]$^-$: 758.23; found m/z 758.37.

4.2.12. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-4-*O*-(3-thienylcarbamoyl)-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (15f)

Compound **11** (20.0 mg, 30 μmol) was dissolved in dry pyridine (0.5 mL). 3-Thienyl isocyanate (12 μL, 0.1 mmol) and DMAP (1.0 mg, 8.0 μmol) were added. The reaction mixture was stirred for 24 h and then water (0.1 mL) was added. After removal of the solvents under reduced pressure, the crude product was purified by chromatography on silica gel (10% gradient of MeOH in DCM) to yield **15f** (8.0 mg, 35%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.25, 1.38 (2s, 6H, C(CH_3)$_2$), 1.87–1.90 (m, 4H, H-3a, NHAc), 2.76 (dd, J = 4.3, 12.8 Hz, 1H, H-3b), 3.48–3.63 (m, 1H, H-9a), 3.81–3.94 (m, 1H, H-9b), 4.10 (d, J = 10.4 Hz, 1H, H-7), 4.27–4.36 (m, 3H, H-5, H-6, H-8), 4.58, 4.70 (A, B of AB, J = 11.6 Hz, 2H, CH_2Ar), 5.15–5.29 (m, 1H, H-4), 6.93 (d, J = 5.4 Hz, 1H, CH_{ar}), 7.02–7.08 (m, 1H, CH_{ar}), 7.09–7.19 (m, 2H, CH_{ar}), 7.20–7.25 (m, 2H, CH_{ar}), 7.31 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.8 (NHAc), 25.5, 27.7 (C(CH_3)$_2$), 38.3 (C-3), 42.5 (C-9), 52.1 (C-5), 60.7 (CH_2Ar), 70.9, 71.8 (C-4, C-7), 76.1, 76.8 (C-6, C-8), 101.8 (C-2), 108.1 (C(CH_3)$_2$), 109.3, 117.5, 117.6, 121.8, 125.3, 125.4, 125.6, 126.0, 129.5, 130.6, 134.1, 138.0, 138.6, 150.5, 152.5 (16C, C-Ar), 155.2 (NC(O)O), 169.4, 173.6, 173.9 (3CO). ESI-MS calcd for $C_{33}H_{33}ClF_2N_3O_{10}S$ [M−H]$^-$: 736.15; found m/z 736.26.

4.2.13. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-4-*O*-(2-(2-thienyl)ethylcarbamoyl)-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (15g)

Compound **11** (28.0 mg, 40 μmol) was dissolved in dry pyridine (0.5 mL). 2-(2-Thienyl)ethyl isocyanate (27.5 mg, 0.18 mmol) and DMAP (1.0 mg, 8.0 μmol) were added. The reaction mixture was stirred for 24 h at 45 °C, then cooled to rt, and treated with water (0.1 mL). After removal of the solvents under reduced pressure, the crude product was purified by chromatography on silica gel (10% gradient of MeOH in DCM) to yield **15g** (16 mg, 37%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.20, 1.27 (2s, 6H, C(CH_3)$_2$), 1.71 (t, J = 12.3 Hz, 1H, H-3a), 1.81 (s, 3H, NHAc), 2.62 (d, J = 4.8, 12.3 Hz, 1H, H-3b), 2.81–2.91 (m, 2H, CH_2), 3.15–3.22 (m, 2H, CH_2), 3.95–4.04 (m, 3H, H-7, H-9a, H-9b), 4.16–4.25 (m, 2H, H-5, H-8), 4.46–4.55 (m, 1H, H-6), 4.64, 4.88 (A, B of AB, J = 12.8 Hz, 2H, CH_2Ar), 4.98–5.08 (m, 1H, H-4), 6.64–6.74 (m, 1H, CH_{ar}), 6.75–6.81 (m, 1H, CH_{ar}), 6.84–6.93 (m, 1H, CH_{ar}), 6.94–7.00 (m, 1H, CH_{ar}), 7.07 (d, J = 4.9 Hz, 1H, CH_{ar}), 7.10–7.20 (m, 1H, CH_{ar}), 7.23, 7.74 (AA′, BB′ of AA′BB′, J = 8.3 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 23.0 (NHAc), 25.9, 27.7 (C(CH_3)$_2$), 31.0 (CH_2), 38.6 (C-3), 43.5 (C-9), 49.9 (CH_2), 52.1 (C-5), 60.8 (CH_2Ar), 71.0 (C-7), 75.8 (C-4), 76.1 (C-8), 76.9 (C-6), 101.6 (C-2), 109.4 (C(CH_3)$_2$), 117.7, 124.7, 126.3, 127.9, 129.5, 130.4, 130.6, 134.0, 138.7, 139.0, 140.0, 142.4, 142.5, 148.6, 150.5, 152.4 (16 C-Ar), 158.4 (NC(O)O), 169.4, 173.5, 173.6 (3CO). ESI-MS calcd for $C_{35}H_{38}ClF_2N_3O_{10}S$ [M+Na]$^+$: 788.19; found m/z 788.42.

4.2.14. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-4-*O*-((2-methylfuran-3-yl)carbamoyl)-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (15h)

Compound **11** (37.0 mg, 50 μmol) was dissolved in dry pyridine (0.5 mL). 2-Methylfuran-3-yl isocyanate (22.0 mg, 0.18 mmol) and DMAP (1.0 mg, 8.0 μmol) were added. The reaction mixture was stirred for 24 h and then water was added. After removal of the solvents under reduced pressure, the crude product was purified by chromatography on silica gel (10% gradient of MeOH in DCM) to yield **15h** (11.0 mg, 28%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.20, 1.33 (2s, 6H, C(CH_3)$_2$), 1.72–1.82 (m, 1H, H-3a), 1.85 (s, 3H, NHAc), 2.08 (s, 3H, CH_3), 2.68 (dd, J = 4.4, 12.8 Hz, 1H, H-3b), 3.40–3.53 (m, 1H, H-9a), 3.84 (d, J = 12.8 Hz, 1H, H-9b), 4.04 (d, J = 10.2 Hz, 1H, H-7), 4.20–4.29 (m, 3H, H-5, H-6, H-8), 4.51, 4.63 (A, B of AB, J = 11.7 Hz, 2H, CH_2Ar), 5.11 (td, J = 4.2, 10.7, 11.1 Hz, 1H, H-4), 6.36 (s, 1H, CH_{ar}), 6.97–7.20 (m, 4H, CH_{ar}), 7.27, 7.78 (AA′, BB′ of AA′BB′, J = 8.4 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 11.2 (CH_3), 22.9 (NHAc), 25.6, 27.6 (C(CH_3)$_2$), 38.3 (C-3), 42.5 (C-9), 52.2 (C-5), 60.7 (CH_2Ar), 70.9 (C-7), 71.9 (C-4), 76.1 (C-8), 76.9 (C-6), 101.8 (C-2), 109.3 (C(CH_3)$_2$), 111.4, 117.5, 117.6, 120.4, 125.5, 126.0, 126.4, 129.5, 130.6, 134.2, 138.6, 138.9, 140.8, 150.5 (16C, C-Ar), 156.4 (NC(O)O), 169.4, 173.6 (3C, 3CO). ESI-MS calcd for $C_{34}H_{36}ClF_2N_3O_{11}$ [M+Na]$^+$: 758.18; found m/z 758.42.

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4.2.15. Sodium [(2,3-difluorobenzyl) 5-acetamido-4-*O*-acetyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (16a)

Compound **15a** (11.0 mg, 20 μmol) was dissolved in 80% aq AcOH (1.5 mL) and stirred for 3 h at 60 °C. Then the reaction mixture was cooled to rt and neutralized with 10% aq NaOH. After removal of the solvents under reduced pressure the crude product was purified on RP-18 (10% gradient of MeOH in water) to yield **16a** (8.5 mg, 80%). $[\alpha]_D^{20}$ −32.8 (*c* 0.24, MeOH); ^{1}H NMR (500 MHz, D_2O): δ 1.79 (t, *J* = 12.1 Hz, 1H, H-3a), 1.91 (s, 3H, NHAc), 2.03 (s, 3H, OAc), 2.75 (dd, *J* = 4.8, 12.4 Hz, 1H, H-3b), 3.42 (dd, *J* = 7.9, 14.2 Hz, 1H, H-9a), 3.52 (d, *J* = 10.0 Hz, 1H, H-7), 3.69–3.80 (m, 2H, H-8, H-9b), 3.91 (d, *J* = 11.7 Hz, 1H, H-6), 4.04 (t, *J* = 10.3 Hz, 1H, H-5), 4.62, 4.87 (A, B of AB, *J* = 11.8 Hz, 2H, CH_2Ar), 4.91 (td, *J* = 4.9, 11.5 Hz, 1H, H-4), 7.08 (dt, *J* = 8.4, 13.1 Hz, 2H, CH_{ar}), 7.17 (t, *J* = 6.7 Hz, 1H, CH_{ar}), 7.49, 7.72 (AA′, BB′ of AA′BB′, *J* = 8.5 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, D_2O): δ 20.2 (OAc), 21.8 (NHAc), 37.3 (C-3), 42.6 (C-9), 49.3 (C-5), 60.6 (CH_2Ar), 69.6, 70.2, 70.8, 72.1 (C-4, C-6, C-7, C-8), 101.1 (C-2), 117.0, 124.3, 125.8, 126.5, 128.6, 128.8, 132.1, 137.5, 138.0, 147.5, 149.0, 149.5 (12 C-Ar), 167.0, 172.8, 173.1, 174.5 (4CO). HRMS calcd for $C_{27}H_{29}ClF_2N_2O_9$ [M+Na]$^+$: 637.1379; found *m*/*z* 637.1362.

4.2.16. Sodium [(2,3-difluorobenzyl) 5-acetamido-4-*O*-benzoyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (16b)

Prepared from **15b** (7.1 mg, 10 μmol) according to the procedure described for **16a**. Purification on RP-18 (10% gradient of MeOH in water) yielded **16b** (4.1 mg, 60%). $[\alpha]_D^{20}$ −7.4 (*c* 0.28, MeOH); ^{1}H NMR (500 MHz, D_2O): δ 1.80 (s, 3H, NHAc), 1.96 (t, *J* = 12.0 Hz, 1H, H-3a), 2.90 (dd, *J* = 4.9, 12.4 Hz, 1H, H-3b), 3.43 (dd, *J* = 8.0, 14.3 Hz, 1H, H-9a), 3.56 (d, *J* = 8.7 Hz, 1H, H-7), 3.73–3.83 (m, 2H, H-8, H-9b), 4.00 (dd, *J* = 1.8, 10.6 Hz, 1H, H-6), 4.24 (t, *J* = 10.3 Hz, 1H, H-5), 4.66, 4.81 (A, B of AB, *J* = 11.9 Hz, 2H, CH_2Ar), 5.13 (ddd, *J* = 4.8, 10.2, 11.6 Hz, 1H, H-4), 7.01–7.17 (m, 2H, CH_{ar}), 7.20 (t, *J* = 6.9 Hz, 1H, CH_{ar}), 7.46–7.56 (m, 4H, CH_{ar}), 7.65 (t, *J* = 7.5 Hz, 1H, CH_{ar}), 7.74, 7.96 (AA′, BB′ of AA′BB′, *J* = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, D_2O): δ 21.7 (NHAc), 37.4 (C-3), 42.6 (C-9), 49.4 (C-5), 60.7 (CH_2Ar), 69.6 (C-7), 70.2 (C-8), 71.7 (C-4), 72.2 (C-6), 100.0 (C-2), 117.0, 117.2, 125.8, 128.7, 128.8, 128.9, 129.4, 132.1, 133.9, 137.5 (18C, C-Ar), 172.9, 174.3 (4C, 4CO). HRMS calcd for $C_{32}H_{30}ClF_2N_2NaO_{10}$ [M+Na]$^+$: 721.1345; found *m*/*z* 721.1353.

4.2.17. Sodium [(2,3-difluorobenzyl) 5-acetamido-4-*O*-benzyl-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (16c)

Prepared from **15c** (6.8 mg, 10 μmol) according to the procedure described for **16a**. Purification on RP-18 (10% gradient of MeOH in water) yielded **16c** (2.0 mg, 36%). $[\alpha]_D^{20}$ −8.4 (*c* 0.16, MeOH); ^{1}H NMR (500 MHz, D_2O): δ 1.55 (t, *J* = 12.0 Hz, 1H, H-3a), 1.77 (s, 3H, NHAc), 2.86 (dd, *J* = 4.6, 12.4 Hz, 1H, H-3b), 3.27–3.39 (m, 2H, H-7, H-9a), 3.42–3.51 (m, 1H, H-4), 3.60–3.69 (m, 3H, H-6, H-8, H-9b), 3.79 (t, *J* = 10.2 Hz, 1H, H-5), 4.39, 4.55 (A, B of AB, *J* = 11.8 Hz, 2H, CH_2Ar), 4.61, 4.70 (A′, B′ of A′B′, *J* = 11.6 Hz, 2H, CH_2Ar), 7.00–7.12 (m, 3H, CH_{ar}), 7.20–7.35 (m, 5H, CH_{ar}), 7.41, 7.62 (AA′, BB′ of AA′BB′, *J* = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, D_2O): δ 21.9 (NHAc), 37.8 (C-3), 49.5 (C-9), 50.0 (C-5), 69.7, 70.2, 71.2 (C-7, C-8, CH_2Ar), 72.6 (C-6), 75.6 (C-4), 101.4 (C-2), 126.0, 128.3, 128.6, 128.7, 128.8, 132.1, 136.7 (18C, C-Ar), 170.0, 172.9, 174.5 (3CO). HRMS calcd for $C_{32}H_{32}ClF_2N_2NaO_9$ [M+H]$^+$: 685.1741; found *m*/*z* 685.1745.

4.2.18. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-4-*O*-phenylcarbamoyl-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (16d)

Prepared from **15d** (10 mg, 10 μmol) according to the procedure described for **16a**. Purification on RP-18 (10% gradient of MeOH in water) yielded **16d** (1.5 mg, 15%). $[\alpha]_D^{20}$ −1.0 (*c* 0.1, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 1.78 (s, 3H, NHAc), 1.88–1.99 (m, 1H, H-3a), 2.68 (d, *J* = 9.1 Hz, 1H, H-3b), 3.33–3.49 (m, 1H, H-9a), 3.60 (d, *J* = 10.7 Hz, 1H, H-9b), 3.74 (d, *J* = 5.4 Hz, 1H, H-7), 4.14 (d, *J* = 2.6 Hz, 1H, H-8), 4.19–4.46 (m, 2H, H-5, H-6), 4.57, 4.79 (A, B of AB, *J* = 11.2 Hz, 2H, CH_2Ar), 5.26 (td, *J* = 4.9, 10.7 Hz, 1H, H-4), 6.95 (t, *J* = 7.2 Hz, 1H, CH_{ar}), 7.13 (d, *J* = 6.0 Hz, 2H, CH_{ar}), 7.19 (t, *J* = 7.7 Hz, 3H, CH_{ar}), 7.28–7.35 (m, 2H, CH_{ar}), 7.41, 7.82 (AA′, BB′ of AA′BB′, *J* = 8.3 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.8 (NHAc), 38.9 (C-3), 44.2 (C-9), 51.2 (C-5), 60.4 (CH_2Ar), 69.4 (C-7), 70.8 (C-4), 72.5 (C-8), 73.3 (C-6), 103.0 (C-2), 117.6, 117.7, 119.8, 124.2, 125.6, 126.3, 128.1, 129.4, 129.8, 130.3, 133.4, 139.1, 139.9, 150.6 (18C, C-Ar), 154.9 (NC(O)O), 169.9, 174.3, 177.6 (3CO). ESI-MS calcd for $C_{32}H_{32}ClF_2N_3O_{10}$ [M+Na]$^+$: 714.16; found *m*/*z* 714.30.

4.2.19. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-4-*O*-phenylethylcarbamoyl-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (16e)

Prepared from **15e** (7.3 mg, 10 μmol) according to the procedure described for **16a**. Purification on RP-18 (10% gradient of MeOH in water) yielded **16e** (1.5 mg, 20%). $[\alpha]_D^{20}$ −5.6 (*c* 0.11, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 1.71–1.83 (m, 1H, H-3a), 1.87 (s, 3H, NHAc), 2.38–2.51 (m, 1H, H-3b), 2.53–2.68 (m, 2H, CH_2), 3.05–3.18 (m, 2H, CH_2), 3.31–3.47 (m, 2H, H-8, H-9a), 3.48–3.65 (m, 2H, H-7, H-9b), 4.05–4.29 (m, 2H, H-5, H-6), 4.46 (A, B of AB, *J* = 11.4 Hz, 2H, CH_2Ar), 5.04 (dt, *J* = 5.3, 10.5, 11.0 Hz, 1H, H-4), 6.93–7.19 (m, 8H, CH_{ar}), 7.32, 7.72 (AA′, BB′ of AA′BB′, *J* = 8.2 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.8 (NHAc), 37.0 (C-3), 39.0, 43.4 (2 CH_2), 44.6 (C-9), 51.2 (C-5), 60.4 (CH_2Ar), 70.0 (C-4), 70.9, 72.1 (C-7, C-8), 72.3 (C-6), 102.8 (C-2), 117.6, 125.3, 126.3, 126.6, 127.2, 127.3, 129.5, 129.8, 130.2, 130.3, 133.7, 134.2, 138.7, 139.0, 140.4, 152.5 (18C, C-Ar), 158.0 (NC(O)O), 169.9, 174.0, 175.9 (3CO). HRMS calcd for $C_{34}H_{36}ClF_2N_3O_{10}$ [M+Na]$^+$: 764.1774; found *m*/*z* 764.1791.

4.2.20. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-4-*O*-(3-thienylcarbamoyl)-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (16f)

Prepared from **15f** (8.0 mg, 10 μmol) according to the procedure described for **16a**. Purification on RP-18 (10% gradient of MeOH in water) yielded **16f** (2.1 mg, 31%). $[\alpha]_D^{20}$ −17.8 (*c* 0.5, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 1.76 (t, *J* = 11.9 Hz, 1H, H-3a), 1.88 (s, 3H, NHAc), 3.02 (dd, *J* = 5.0, 12.1 Hz, 1H, H-3b), 3.44 (dd, *J* = 1.7, 9.0 Hz, 1H, H-7), 3.48 (dd, *J* = 7.7, 13.7 Hz, 1H, H-9a), 3.76 (dd, *J* = 3.1, 13.7 Hz, 1H, H-9b), 3.84 (dd, *J* = 1.3, 10.5 Hz, 1H, H-6), 3.97–4.08 (m, 2H, H-5, H-8), 4.66, 4.92 (A, B of AB, *J* = 12.2 Hz, 2H, CH_2Ar), 4.98 (td, *J* = 5.0, 11.0 Hz, 1H, H-4), 6.94 (d, *J* = 4.9 Hz, 1H, CH_{ar}), 7.04–7.11 (m, 2H, CH_{ar}), 7.17 (s, 1H, CH_{ar}), 7.23 (dd, *J* = 3.2, 5.1 Hz, 1H, CH_{ar}), 7.25–7.32 (m, 1H, CH_{ar}), 7.43, 7.80 (AA′, BB′ of AA′BB′, *J* = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.6 (NHAc), 39.7 (C-3), 44.4 (C-9), 51.7 (C-5), 60.3 (CH_2Ar), 71.4 (C-8), 72.2 (C-7), 72.5 (C-4), 74.0 (C-6), 108.0 (C-2), 108.1, 117.0, 121.8, 122.4, 125.2, 125.4, 126.3, 129.7, 130.1, 134.4, 138.6 (16C, C-Ar), 152.4 (NC(O)O), 174.9 (3C, 3CO). HRMS calcd for $C_{30}H_{30}ClF_2N_3O_{10}S$ [M−H]$^-$: 720.1206; found *m*/*z* 720.1209.

4.2.21. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-4-*O*-(2-(2-thienyl)ethylcarbamoyl)-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (16g)

Prepared from **15g** (16.0 mg, 20 μmol) according to the procedure described for **16a**. Purification on RP-18 (10% gradient of MeOH in water) yielded **16g** (1.5 mg, 10%). $[\alpha]_D^{20}$ −5.9 (*c* 0.18, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 1.73–1.83 (m, 4H, H-3a, NHAc), 2.54 (dd, *J* = 5.0, 13.0 Hz, 1H, H-3b), 2.85 (t, *J* = 7.0 Hz, 2H, CH_2), 3.16–3.22 (m, 2H, CH_2), 3.28 (dd, *J* = 6.4, 14.0 Hz, 1H, H-9a),

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3.45 (dd, J = 5.0, 14.0 Hz, 1H, H-9b), 3.66 (d, J = 5.6 Hz, 1H, H-7), 4.00–4.13 (m, 2H, H-5, H-8), 4.20 (d, J = 10.6 Hz, 1H, H-6), 4.50, 4.69 (A, B of AB, J = 11.7 Hz, 2H, CH_2Ar), 5.07 (td, J = 4.9, 10.9 Hz, 1H, H-4), 6.71 (d, J = 3.0 Hz, 1H, CH_{ar}), 6.79–6.85 (m, 1H, CH_{ar}), 7.05–7.19 (m, 4H, CH_{ar}), 7.36, 7.78 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.9 (NHAc), 30.9 (CH_2), 43.5 (C-3), 44.0 (C-9), 46.7 (CH_2), 51.2 (C-5), 60.5 (CH_2Ar), 69.2, 70.6, 72.3 (C-4, C-7, C-8), 73.9 (C-6), 103.0 (C-2), 117.6, 117.7, 124.7, 125.7, 126.1, 126.3, 127.9, 129.8, 130.4, 133.3, 139.2, 142.4 (16C, C-Ar), 157.9 (NC(O)O), 169.8, 174.2, 174.6 (3CO). ESI-MS calcd for $C_{32}H_{34}ClF_2N_3O_{10}S$ [M+Na]$^+$: 748.14; found m/z 748.29.

4.2.22. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-4-*O*-((2-methylfuran-3-yl)carbamoyl)-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (16h)

Prepared from **15h** (11.0 mg, 10 μmol) according to the procedure described for **16a**. Purification on RP-18 (10% gradient of MeOH in water) yielded **16h** (2.4 mg, 25%). $[\alpha]_D^{20}$ −2.4 (*c* 0.37, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 1.71 (dd, J = 7.9, 11.6 Hz, 1H, H-3a), 1.82 (s, 3H, NHAc), 2.07 (s, 3H, CH_3), 2.50 (dd, J = 5.1, 12.3 Hz, 1H, H-3b), 3.31 (d, J = 9.4 Hz, 1H, H-7), 3.44 (dd, J = 6.5, 13.8 Hz, 1H, H-9a), 3.71 (dd, J = 2.6, 13.6 Hz, 1H, H-9b), 3.84–3.97 (m, 1H, H-8), 4.06 (t, J = 11.5 Hz, 1H, H-6), 4.18 (t, J = 10.6 Hz, 1H, H-5), 4.45, 4.69 (A, B of AB, J = 11.2 Hz, 2H, CH_2Ar), 5.11–5.21 (m, 1H, H-4), 6.35 (s, 1H, CH_{ar}), 7.00–7.06 (m, 2H, CH_{ar}), 7.11 (s, 1H, CH_{ar}), 7.18 (s, 1H, CH_{ar}), 7.37, 7.74 (AA′, BB′ of AA′BB′, J = 8.4 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 11.3 (CH_3), 22.9 (NHAc), 39.3 (C-3), 44.1 (C-9), 60.4 (CH_2Ar), 67.5, 70.2, 71.0 (C-4, C-7, C-8), 72.0 (C-6), 101.9 (C-2), 111.4, 112.2, 117.2, 125.4, 126.7, 129.7, 130.2, 130.3, 134.1, 138.6, 138.8, 140.8, 143.5 (16C, C-Ar), 156.4 (NC(O)O), 169.8, 176.7, 180.5 (3CO). HRMS calcd for $C_{31}H_{32}ClF_2N_3O_{11}$ [M+Na]$^+$: 718.1592; found m/z 718.1584.

4.2.23. Methyl [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-α-D-*manno*-2,4-nonudiulopyranosid]onate (17)

Compound **10** (63.0 mg, 10 μmol) was dissolved in dry DCM (2.5 mL). Pyridinium dichromate (26.0 mg, 70 μmol) was added followed by the addition of acetic anhydride (28 μL, 0.3 mmol). The reaction mixture was stirred at rt for 4 h. After addition of 2-propanol (1 mL) the mixture was co-evaporated three times with toluene. Purification by chromatography on silica gel (EtOAc) yielded **17** (20 mg, 30%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.37, 1.52 (2s, 6H, C(CH_3)$_2$), 2.17 (s, 3H, NHAc), 2.94 (d, J = 15.2 Hz, 1H, H-3a), 3.29 (d, J = 15.0 Hz, 1H, H-3b), 3.82 (s, 3H, OMe), 3.85–3.96 (m, 1H, H-9a), 4.02 (dt, J = 6.4, 13.0 Hz, 1H, H-9b), 4.27 (d, J = 6.4 Hz, 1H, H-7), 4.38–4.52 (m, 2H, H-5, H-6), 4.53–4.59 (m, 1H, H-8), 4.64, 5.07 (A, B of AB, J = 11.5 Hz, 2H, CH_2Ar), 6.06 (d, J = 6.9 Hz, 1H, NH), 6.97–7.21 (m, 4H, NH, CH_{ar}), 7.40, 7.76 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, $CDCl_3$): δ 23.1 (NHAc), 25.4, 26.9 (C(CH_3)$_2$), 44.3 (C-3), 47.6 (C-9), 53.7 (C-5), 56.3 (OMe), 60.4 (CH_2Ar), 73.2 (C-7), 74.6 (C-8), 75.3 (C-6), 99.8 (C-2), 109.1 (C(CH$_3$)$_2$), 117.2, 124.1, 124.2, 125.2, 125.9, 126.0, 128.5, 128.6, 128.8, 132.7, 137.8 (12C, C-Ar), 167.2, 170.7, 173.0, 200.1 (4CO). ESI-MS calcd for $C_{29}H_{31}ClF_2N_2O_9$ [M+Na]$^+$: 647.16; found m/z 647.13.

4.2.24. Methyl [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-4-methyl-D-*glycero*-α-D-*talo*-nonulopyranosid]onate (18)

$ZrCl_4$ (50.0 mg, 0.13 mmol, 4.0 equiv) was dried for 30 min at 30 °C under high vacuum. After addition of dry THF (1.7 mL), the suspension was heated up to 50 °C for 20 min. Afterwards the colorless solution was cooled to −54 °C. MeLi (1 M in hexane, 0.5 mL) was added dropwise and the pale yellow solution was warmed up to 0 °C and stirring was continued for 30 min. After cooling to −78 °C, a solution of **17** (20.0 mg, 30 μmol) in THF (0.5 mL) was added. The reaction mixture was stirred for 3 h and then allowed to warm to 0 °C. After addition of semi-satd aq NH_4Cl (0.5 mL), the reaction mixture was extracted with DCM (5 mL). The combined organic layers were dried over Na_2SO_4, filtered, and the solvents were evaporated. Afterwards the crude mixture was dissolved in 80% aq AcOH (1 mL) and stirred for 3 h at 60 °C. After removal of the solvents under reduced pressure, the pure product **18** was obtained by chromatography on silica gel (1% gradient of MeOH in DCM) (6.0 mg, 32% + 2.0 mg, 11% (*S*)-stereoisomer). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.24 (s, 3H, CH_3), 2.07 (s, 3H, NHAc), 2.96 (d, J = 15.0 Hz, 1H, H-3a), 3.35 (d, J = 15.0 Hz, 1H, H-3b), 3.48 (d, J = 8.9 Hz, 1H, H-7), 3.73 (dd, J = 6.9, 13.1 Hz, 1H, H-9a), 3.79 (s, 3H, OMe), 3.82–3.90 (m, 2H, H-6, H-9b), 4.09–4.18 (m, 1H, H-8), 4.68–4.80 (m, 2H, H-5, CH_2Ar), 4.95 (B of AB, J = 11.7 Hz, 1H, CH_2Ar), 6.55 (d, J = 6.6 Hz, 1H, NHAc), 7.00–7.18 (m, 4H, NH, CH_{ar}), 7.39, 7.74 (AA′, BB′ of AA′BB′, J = 8.5 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, $CDCl_3$): δ 23.0 (NHAc), 29.0 (CH_3), 45.2 (C-9), 47.5 (C-3), 52.8 (C-5), 53.6 (OMe), 69.7 (C-8), 70.5 (C-7), 71.5 (C-6), 98.1 (C-2), 122.8, 128.5, 128.6, 129.0, 129.2 (12C, C-Ar), 178.1 (3C, 3CO). ESI-MS calcd for $C_{30}H_{35}ClF_2N_2O_9$ [M+Na]$^+$: 663.19; found m/z 663.05.

4.2.25. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-4-methyl-D-*glycero*-α-D-*talo*-nonulopyranosid]onate (19)

Compound **18** (6.0 mg, 10 μmol) was dissolved in THF (1.0 mL) and LiOH (9.0 mg, 0.4 mmol) in water (1.0 mL) was added. The mixture was stirred at rt for 4 h and neutralized with 7% aq HCl. After removal of the solvents under reduced pressure, the pure product **19** (2.0 mg, 34%) was obtained by chromatography on RP-8 (5% gradient of MeOH in water) followed by Dowex 50X8 ion-exchange and P2 size exclusion chromatography. $[\alpha]_D^{20}$ −6.3 (*c* 0.26, MeOH); ^{1}H NMR (500 MHz, D_2O): δ 1.19 (s, 3H, CH_3), 1.79 (d, J = 14.1 Hz, 1H, H-3a), 2.02 (s, 3H, NHAc), 2.60 (d, J = 14.1 Hz, 1H, H-3b), 3.45 (dd, J = 7.3, 13.6 Hz, 1H, H-9a), 3.50 (dd, J = 1.9, 8.6 Hz, 1H, H-7), 3.65–3.83 (m, 2H, H-8, H-9b), 3.88 (d, J = 10.6 Hz, 1H, H-5), 4.31 (dd, J = 2.0, 10.6 Hz, 1H, H-6), 4.56, 4.76 (A, B of AB, J = 12.0 Hz, 2H, CH_2Ar), 7.03–7.23 (m, 3H, CH_{ar}), 7.53, 7.76 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, D_2O): δ 21.8 (NHAc), 26.0 (CH_3), 42.6 (C-9), 44.8 (C-3), 52.1 (C-5), 60.1 (CH_2Ar), 70.3, 70.5, 70.6, 71.4 (C-4, C-6, C-7, C-8), 100.4 (C-2), 117.0, 124.3, 125.9, 126.7, 128.7, 128.8, 132.2, 137.5 (12C, C-Ar), 170.0, 174.4, 174.6 (3CO). HRMS calcd for $C_{26}H_{30}ClF_2N_2O_9$ [M+Na]$^+$: 585.1427; found m/z 585.1439.

4.2.26. Methyl [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-7,8-*O*-isopropylidene-D-*glycero*-α-D-*talo*-nonulopyranosid]onate (20)

$BH_3{\cdot}NH_3$ (7.0 mg, 0.22 mmol) was dissolved in MeOH (0.5 mL) at 0 °C followed by the addition of **17** (30.0 mg, 50 μmol) in MeOH (0.5 mL). After stirring for 2 h at 0 °C, the solvent was evaporated under reduced pressure and the crude product was purified by chromatography on silica gel (2% gradient of 2-PrOH in DCM/MeOH 10:1) to yield **20** (11.0 mg, 36%) as a white solid. ^{1}H NMR (500 MHz, CD_3OD): δ 1.30, 1.48 (2s, 6H, C(CH_3)$_2$), 1.88 (dd, J = 2.0, 13.7 Hz, 1H, H-3a), 1.95 (s, 3H, NHAc), 2.62 (dd, J = 3.9, 13.7 Hz, 1H, H-3b), 4.02 (d, J = 3.8 Hz, 1H, H-4), 4.07 (t, J = 6.2 Hz, 2H, H-9a, H-9b), 4.13 (dd, J = 2.6, 10.7 Hz, 1H, H-5), 4.20 (d, J = 6.9 Hz, 1H, H-7), 4.37 (d, J = 10.7 Hz, 1H, H-6), 4.43 (A of AB, J = 11.4 Hz, 1H, CH_2Ar), 4.47 (tt, J = 4.5, 8.8 Hz, 1H, H-8), 4.90 (B of AB, J = 11.4 Hz, 1H, CH_2Ar), 7.04–7.13 (m, 1H, CH_{ar}), 7.13–7.22 (m, 2H, CH_{ar}), 7.44, 7.82 (AA′, BB′ of AA′BB′, J = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.7 (NHAc), 25.8, 27.1 (C(CH_3)$_2$), 40.8

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(C-3), 41.9 (C-9), 50.1 (C-5), 52.7 (OMe), 60.5 (CH_2Ar), 66.3 (C-4), 71.5 (C-7), 76.3 (C-6), 77.7 (C-8), 98.9 (C-2), 110.1 ($C(CH_3)_2$), 117.8, 125.4, 126.8, 128.3, 129.7, 130.1, 130.2, 134.3, 138.7 (12C, C-Ar), 169.3, 171.4, 173.1 (3CO). ESI-MS calcd for $C_{29}H_{33}Cl_2F_2N_2O_9$ $[M+Na]^+$: 649.18; found *m*/*z* 649.22.

4.2.27. Methyl [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*talo*-nonulopyranosid]onate (21)

Compound **20** (20.0 mg, 30 μmol) was dissolved in 80% aq AcOH (1.5 mL) and stirred for 3 h at 60 °C. Then, the reaction mixture was cooled to rt and neutralized with 10% aq NaOH. After evaporation of the solvents, the crude product was purified by chromatography on silica gel (10% gradient of MeOH in DCM) to yield **21** (17 mg, 90%) as a colorless oil. ^{1}H NMR (500 MHz, CD_3OD): δ 1.89–1.97 (m, 4H, H-3a, NHAc), 2.62 (dd, *J* = 3.4, 13.9 Hz, 1H, H-3b), 3.42 (dd, *J* = 5.1, 13.8 Hz, 1H, H-6), 3.52 (dd, *J* = 7.6, 13.8 Hz, 1H, H-9a), 3.70 (s, 3H, OMe), 3.78 (dd, *J* = 3.3, 13.9 Hz, 1H, H-9b), 4.01–4.11 (m, 3H, H-4, H-5, H-8), 4.35 (d, *J* = 11.4 Hz, 1H, H-7), 4.45, 4.76 (A, B of AB, *J* = 11.8 Hz, 2H, CH_2Ar), 7.00–7.21 (m, 3H, CH_{ar}), 7.41, 7.78 (AA′, BB′ of AA′BB′, *J* = 8.6 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD): δ 22.7 (NHAc), 41.1 (C-3), 44.9 (C-9), 49.8 (C-5), 53.2 (OMe), 60.3 (CH_2Ar), 67.0 (C-4), 71.3 (C-8), 71.8 (C-7), 72.5 (C-6), 99.0 (C-2), 117.9, 118.0, 125.5, 125.6, 126.7, 128.5, 128.6, 129.8, 130.2, 134.6, 138.8 (12C, C-Ar), 169.6, 172.0, 174.3 (3CO). ESI-MS calcd for $C_{26}H_{29}ClF_2N_2O_9$ $[M+Na]^+$: 587.25; found *m*/*z* 587.25.

4.2.28. Sodium [(2,3-difluorobenzyl) 5-acetamido-9-(4-chlorobenzamido)-3,5,9-trideoxy-D-*glycero*-α-D-*talo*-nonulopyranosid]onate (22)

Compound **21** (17.0 mg, 30 μmol) was dissolved in MeOH (3 mL) and 10% aq NaOH (0.1 mL) was added. After 2 h the reaction was neutralized with 7% aq HCl. The solvents were evaporated and the residue was purified by chromatography on RP-8 (10% gradient of MeOH in water) to yield **22** (2.0 mg, 34%). $[\alpha]_D^{20}$ −41.6 (*c* 0.37, MeOH); ^{1}H NMR (500 MHz, D_2O): δ 1.85 (dd, *J* = 2.9, 14.2 Hz, 1H, H-3a), 1.96 (s, 3H, NHAc), 2.62 (dd, *J* = 3.4, 14.2 Hz, 1H, H-3b), 3.44 (dd, *J* = 7.9, 14.2 Hz, 1H, H-9a), 3.49 (dd, *J* = 1.8, 8.9 Hz, 1H, H-7), 3.67–3.79 (m, 2H, H-8, H-9b), 4.00 (dd, *J* = 2.8, 10.7 Hz, 1H, H-5), 4.13 (q, *J* = 2.9 Hz, 1H, H-4), 4.37 (dd, *J* = 1.8, 10.7 Hz, 1H, H-6), 4.53, 4.73 (A, B of AB, *J* = 11.7 Hz, 2H, CH_2Ar), 6.97–7.25 (m, 3H, CH_{ar}), 7.49, 7.73 (AA′, BB′ of AA′BB′, *J* = 8.5 Hz, 4H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 21.8 (NHAc), 39.2 (C-3), 42.6 (C-9), 48.2 (C-5), 60.1 (CH_2Ar), 65.8 (C-4), 69.4 (C-6), 70.2, 70.3 (C-7, C-8), 100.0 (C-2), 117.0, 125.9, 128.7, 128.8, 132.1, 137.5 (12C, C-Ar), 170.0, 174.5 (3C, 3CO). HRMS calcd for $C_{25}H_{27}ClF_2N_2O_9$ $[M+Na]^+$: 595.1273; found *m*/*z* 595.1276.

4.3. Surface plasmon resonance (SPR) analysis

The SPR measurements were performed on a Biacore 3000 surface plasmon resonance-based optical biosensor (Biacore AB, Sweden). Sensor chips (CM5), immobilization kits, maintenance supply, and HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) were purchased from Biacore AB (HBS-EP ready-to-use; degassed and filtered). CM5 chips were preconditioned prior to usage by injecting a series of conditioning solutions. A flow rate of 50 μL/min was used and 2 × 20 μL of 50 mM NaOH, 10 mM HCl, 0.1% SDS, and 100 mM H_3PO_4 were injected. The carboxy groups on the CM5 chip were activated for 10 min with a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.1 M 3-(*N*,*N*-dimethylamino)propyl-*N*-ethylcarbodiimide (EDC) at a flow rate of 10 μL/min. Protein A (P6031) was purchased from Sigma. A sample and a reference surface were prepared sequentially or in parallel. For immobilizing protein A, a stock solution (1 mg/mL in 50 mM phosphate buffer, pH 7.0) was diluted in 10 mM sodium acetate, pH 5.0, to obtain a concentration of 30 μg/mL. This solution was then injected over the activated surface for 10 min at a flow rate of 10 μL/min. Protein A densities around 4'000 to 5'000 RU were achieved. Flow cells were blocked with a 10-min injection of 1 M ethanolamine, pH 8.0. For capturing, $MAG_{d1\text{-}3}$-Fc solution (expressed and purified as described[32]) was diluted to a 30–40 μg/mL concentration using HBS-EP. Afterwards, $MAG_{d1\text{-}3}$-Fc was injected at a flow rate of 1 μL/min for 10 min. Using HBS-EP, the surface was equilibrated overnight at a flow rate of 5 μL/min, achieving densities around 2000 RU. Tenfold dilution series were freshly prepared in eluent buffer immediately before use (→**14**, **16a**–**c**, **19**, and **22**). All binding experiments were conducted at 25 °C at a flow rate of 20 μL/min. The samples were injected over 1 min followed by 1 min dissociation. Each sample was measured with a duplicate of one concentration, using a randomized concentration order. Several buffer samples were injected before the first concentration, and one blank between each concentration, which was used for the double blank referencing during data processing. Double referencing was applied to correct for bulk effects and other systematic artifacts. Data processing and equilibrium binding constant determinations were accomplished with Scrubber (BioLogic Software, Version 1.1 g or 2.0c). Kinetic data were simultaneously fit using Scrubber 2.0c. For the DMSO assay, DMSO (for molecular biology, >99.9%) was purchased from Fluka. The stock solution of the test compounds (→**16d**–**h**) was prepared in DMSO and was kept in glass vials to eliminate contaminations by, for example, softeners. The running buffer was 3% DMSO in HBS-EP. The surface was equilibrated at a flow of 5 μL/min until the baseline was stable. In order to eliminate the influence of DMSO on the signals, a calibration curve was done. Therefore, two solutions were prepared (A = 1 mL running buffer + 50 μL HBS-EP; B = 1 mL running buffer + 1 μL DMSO). Solutions A and B were mixed as indicated in Table 5 and used for calibration. DMSO calibration solutions were injected after five blank injections and before the sample solutions. The test compounds were diluted before measuring with HBS-EP to achieve a content of 3% DMSO. The DMSO calibration was accomplished directly in Scrubber© (version 2.0c).

4.4. Isothermal titration calorimetry

ITC experiments were performed using a VP-ITC instrument from MicroCal, Inc. (Northampton, MA). The measurements were performed at 25 °C. Injections of 5 μL ligand solutions were added from a computer controlled 300 μL microsyringe at an interval of 5 min into the sample cell solution of $MAG_{d1\text{-}3}$-Fc (cell volume 1.4512 mL) with stirring at 307 rpm. A control experiment was performed, where the identical ligand solutions were injected into buffer without protein. The enthalpogram showed negligible heat development, resulting from dilution effects. The assay buffer was HBS-E (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4). The concentration of $MAG_{d1\text{-}3}$-Fc was 48.4 μM, and 500 μM antagonist was injected. The experimental data were fitted to a theoretical titration curve (one site binding model) using *Origin version 7* software (MicroCal), with ΔH (enthalpy change in kcal/mol), K_A (association constant in M^{-1}), and *N* (number of binding sites) as adjustable parameters. The quantity $c = K_A \cdot Mt(0)$, where Mt(0) is the initial macromolecule concentration, is of importance in titra-

Table 5
Calibration solutions

Calibration	1	2	3	4	5
A (μL)	400	300	200	100	0
B (μL)	0	100	200	300	400

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tion microcalorimetry.[31] The experiment was performed with a *c* value of 340. Thermodynamic parameters were calculated from Eq. (1),

$$\Delta G = \Delta H - T\Delta S = RT \ln K_A = -RT \ln K_D \quad (1)$$

where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively, T is the absolute temperature, and R = 1.98 cal/mol/K. For reasons of consistency the values were converted to kJ (1 cal = 4.1868 J).

4.5. HPLC

The concentration of $MAG_{d1\text{-}3}$-Fc was determined via HPLC against a standard curve of BSA at 210 nm using a Beckmann Gold system, with UV detection (210 nm). The column used was Poros R1/10 10 μm (100 × 2 mm, Dr. Maisch HPLC Markensäulen, po10.r1.s1002, Morvay Analytik GmbH). The running buffers were A: H_2O + 0.1% TFA and B: 90% MeCN + 0.09% TFA. All measurements were performed at 75 °C, applying a gradient of 20–90% running buffer B within 20 min at a flow rate of 0.2 mL/min.[44]

Acknowledgments

We are greatly indebted to Dr. Francis Bitsch and Peggy Brunet Lefeuvre, Novartis, for providing a HPLC method for protein concentration determination and for providing the VP-ITC instrument as well as for advices regarding the experimental setup and analyzing the data. In addition, we would like to thank the Swiss National Science Foundation (project 200020-120628), the German Ministry for Education and Research ((BMBF, project 031632A), and the Tönjes-Vogt Foundation (project XXI) for their support of this work.

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MAG-antagonists: Approach towards high-affinity ligands by click-chemistry

This report comprises the optimization approach applied to a novel generation of MAG-antagonists.

The article is a draft version and supposed to be submitted to *ChemMedChem*.
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Work performed by Stefanie Mesch:
Synthesis of ligand **9**, performance and evaluation of Biacore assay.

MAG-antagonists: Approach towards high-affinity ligands by click-chemistry

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Keywords. Carbohydrate-mimetics, Myelin-associated glycoprotein (MAG), Siglecs, Surface plasmon resonance, Thermodynamics of carbohydrate-protein interactions.

Introduction. The central nervous system (CNS) is an inhibitory environment for axon regeneration.[1,2] After injury, nerve strands lack the ability of adhesion due to the presence of several inhibitory proteins.[3] In the last decade, three inhibitors have been identified, one of them being the myelin-associated glycoprotein (MAG)[4]. MAG interacts with several neuronal receptors, such as proteins of the Nogo receptor family and gangliosides, primarily GD1a, GT1b and GQ1bα.[5-8] Although the relative role of Nogo receptors and gangliosides as MAG ligands has yet to be resolved, it is supposed that binding to MAG leads in all cases to the activation of the small kinase RhoA and finally to growth cone collapse.[9]

We focus on the ganglioside mediated cascade, as in certain systems, sialidase treatment resulted in enhanced neural outgrowth.[10] Taking this one step further, blocking MAG with potent glycomimetic antagonists offers a valuable therapeutic approach to enhance axon regeneration. Extensive SAR studies resulted in mimetics with simplified structures and high potency.[11-14] For further affinity enhancement and with respect to selectivity issues, the concept of "fragment assembly" was applied. Here, fragments binding in proximity are linked and benefit results from the multiplied affinities.[15] Shelke *et al*. used a NMR based approach for the identification of second site fragments.[16] The fragments were linked *in situ* applying Cu^{I}-catalyzed Huisgen [3+2] cycloaddition, also known as click-chemistry, yielded a lead compound providing a K_D in the low nanomolar range[17] (Figure 1). In this report we present the optimization of the identified lead by means of medicinal chemistry and the biological evaluation thereof.

Results and Discussion. The influence of a radical on the relaxation-behavior of protons located in close proximity is used by NMR-based screenings to identify weak binders around any ligand's binding site.[18] In the case of MAG, Shelke *et al.* found that 5'-nitroindole is binding near the binding site of sialic acid. After evaluation of the optimal spacer length by *in situ* click experiments, antagonist **1** was synthesized and its affinity was determined (see also Table 1).[17] In a next step this lead was considered for further optimization. As the attachment of the linker on the indole nitrogen led to a decrease in affinity (data not shown), attachment of the linker at 3'-C was maintained.

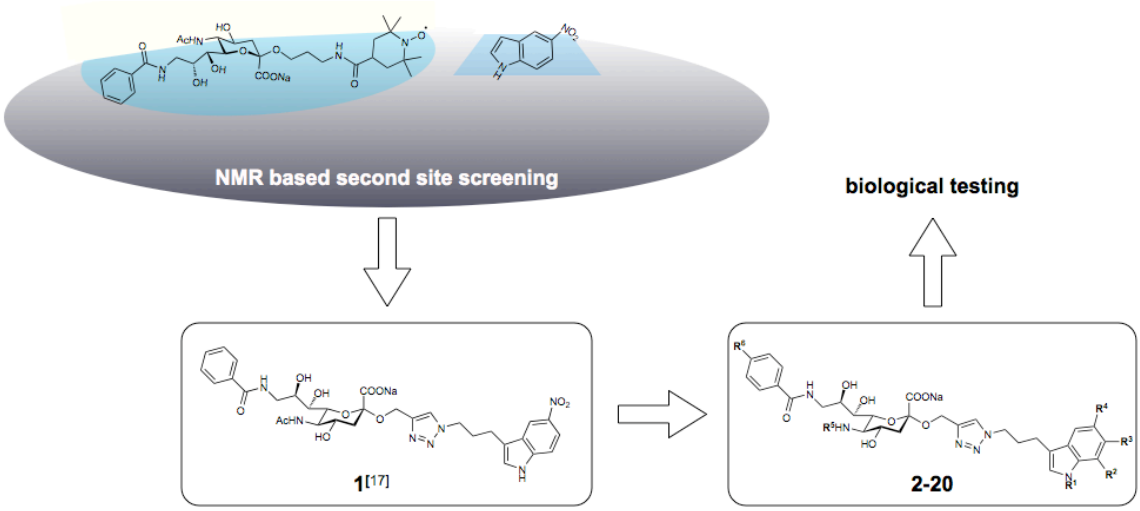
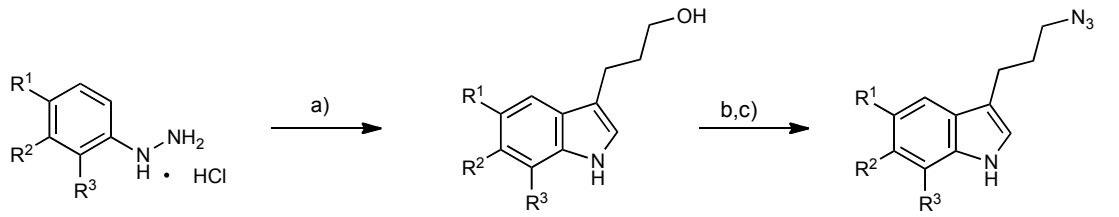


Figure 1. Lead identification by NMR based approach and further optimization.

Here, we examine the influence of various modifications on the indole such as alkylation of the indole nitrogen and alteration of the 5'-, 6'- and 7'-substituents respectively. Apart from elucidating the effects of the electron density on binding affinity, the hydrophobic surface of the indole was enlarged by amides in 6'-position. During the optimization process, lead compound **1** was modified in 5-position of neuraminic acid by inserting fluoroacetate, which was found earlier to improve binding affinity and *p*-chlorobenzamide in 9-position.[14,19] Final combination with miscellaneous substituted indole moieties yielded a library of antagonists **2**-**20**. The binding affinity of the final compounds was elucidated using a surface plasmon resonance based assay, hapten binding assay and ITC. Furthermore, molecular mechanic studies were conducted to rationalize the binding process and the effect of the different substituents was scrutinized by testing the ligands towards a MAG mutant. Finally, the thermodynamic profile of the most potent antagonists was investigated.

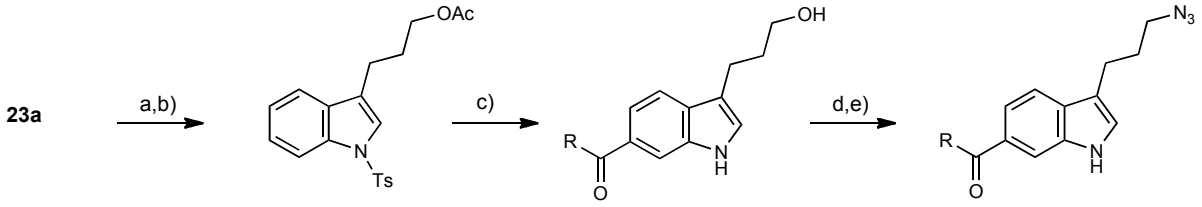
Synthesis. The antagonists **2 - 20** (Figure 1) were obtained by click-chemistry as the final key step. Sialoside **21** (Scheme 4) was synthesized according to a reported procedure[17] and the synthesis of the various indole substituents was accomplished by following different approaches (Scheme 1 - 3). In more detail, 3- and 3,5-substituted indoles were synthesized by reacting hydrazine derivatives **22a - 22k** with 3,4-dihydro-2*H*-pyran under modified Fisher indolization conditions (→**23a - k**).[20] Following, the primary alcohols were converted into the corresponding azides by applying the Appel-reaction.[21,22]



a: R^1, R^2, R^3 = H
b: R^1 = OMe, R^2, R^3 = H
c: R^1 = Cl, R^2, R^3 = H
d: R^1 = F, R^2, R^3 = H
e: R^1, R^2 = H, R^3 = Me
f: R^1 = H, R^2 = Cl, R^3 = H
g: R^1 = isopropyl, R^2, R^3 = H
h: R^1, R^2 = -$(CH_2)_3$-, R^3 = H
i: R^1, R^2 = H, R^3 = Cl
j: R^1 = CN, R^2, R^3 = H
k: R^1 = SO_2Me, R^2, R^3 = H

Scheme 1. a) 3,4-Dihydro-2*H*-pyran, DMAc, 4% aq. H_2SO_4, 100 °C (11–90%); (b) PPh_3, CBr_4, CH_3CN (→**24a - k**); (c) NaN_3, DMF (16-85%, 2 steps).

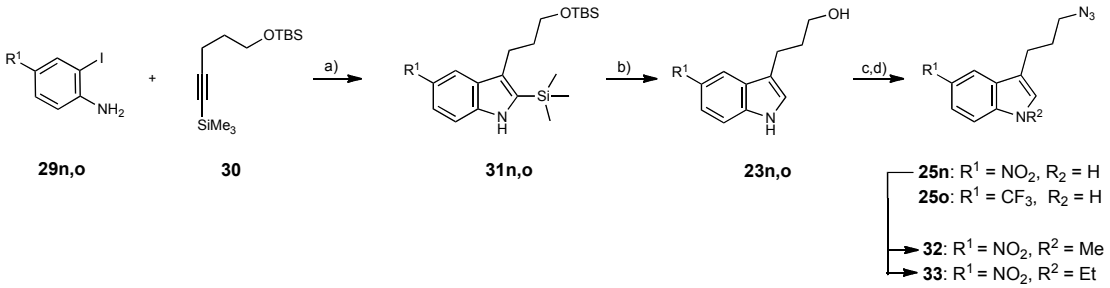
Starting from intermediate **23a**, 3,6-disubstituted indoles were obtained after protection of the free alcohol and the indole-amine by performing Friedel-Crafts acylation (→**23l, m**). Subsequent deprotection and conversion of the primary alcohol into the azide yielded the desired indole derivatives (→**25l, m**) for click-chemistry.



25l: R = cyclopropyl
25m: R = 4-chlorophenyl

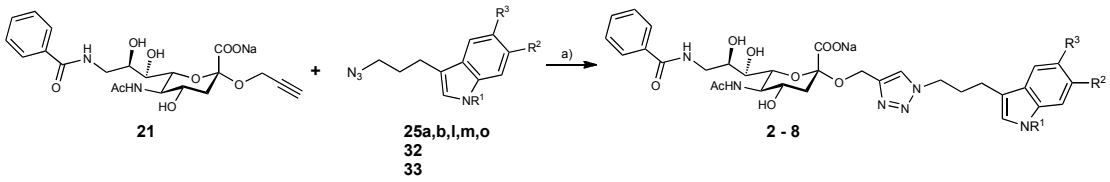
Scheme 2. a) Ac_2O, DMAP, pyridine (→**27:** 92%); b) TsCl, NaH, THF (70%); c) (i) RCOCl, $AlCl_3$, CH_3NO_2, (ii) 6 M NaOH, MeOH (**23l**: 61%; **23m**: 47%). Yields refer to inseparable mixture of 5- and 6- substituted indoles); d) PPh_3, CBr_4, CH_3CN, rt, (→**24l, m**); e) DMF, NaN_3, rt (**25l**: 36%; **25m**: 37%)

In the case of 1,3,5- and 3,5-substituted indoles, having electron-withdrawing substituents in 5'-position (Scheme 3), they were synthesized by reacting 2-iodo-aniline derivatives **29n, o** with **30** under Larock indole conditions[22,23] (→**31n, o**). After removal of the silyl protecting group, the alcohol (→**23n, o**) was converted into the desired azide (→**25n, o**).[17] Starting from **25n** *N*-alkylation was performed using the corresponding alkyl iodides and potassium hydroxide as base to receive **32** and **33**.



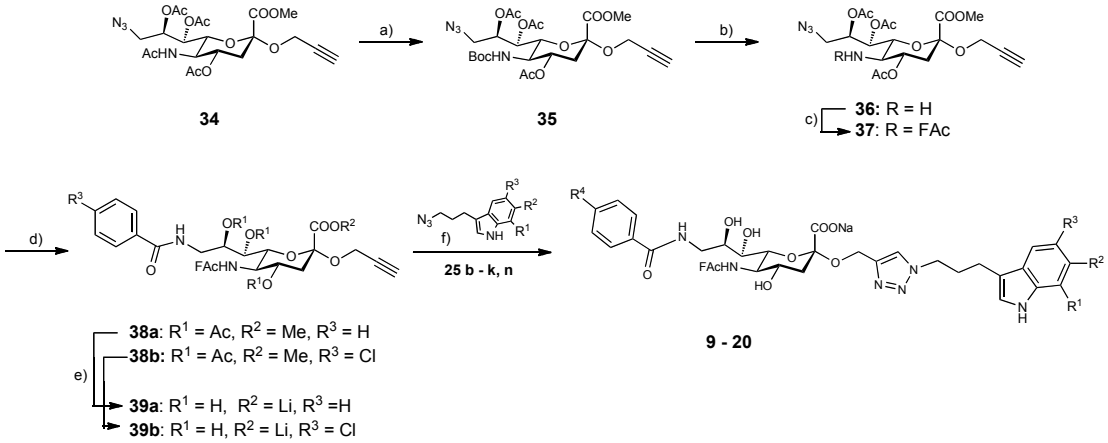
Scheme 3. a) $Pd(OAc)_2$, LiCl, KOAc, DMF, 70 °C; b) 48% HF, CH_3CN, rt (**23n**: 62%; **23o**: 69%, 2 steps); c) PPh_3, CBr_4, CH_3CN (**24n, o**); d) DMF, NaN_3, rt (**25n**: 85%; **25o**: 80%, 2 steps); e) R^2I, KOH, DMF (**32**: 94%; **33**: 92%).

Finally, with sialoside **21** and the various substituted indole derivatives in hand, the final compounds were synthesized applying Cu^I-catalyzed click-conditions as shown in scheme 4.



Scheme 4. a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, H_2O/*t*-BuOH 1:1, rt (**2 - 8:** 47-80%); for R^1, R^2 and R^3 see Table.

Earlier SAR studies showed that introduction of fluoro acetamide in 5-position leads to a distinct improvement of the binding affinity[14,19] and therefore we decided to incorporate this modification into compound **1** (Scheme 5). Intermediate **35** was obtained by cleavage of the *N*-acetate in 5-position followed by Boc-protection to give **36**. After deprotection, selective acylation was performed (→**37**). Afterwards, the azide was transformed into benzylamide or *p*-chlorobenzylamide, respectively, under modified Staudinger conditions (→**38a, b**). After deprotection (→**39a, b**) the test compounds **9 - 20** were obtained by click-reaction with indole derivatives **25b –k, n.**



Scheme 5. a) i. Boc_2O, DMAP, THF; ii. $N_2H_4 \cdot H_2O$, MeOH; iii. Ac_2O, DMAP, pyridine (50%); b) PhOH, TMSCl, DCM, rt (70%); c) FCH_2COCl, NEt_3, DMAP, THF (43%); d) PPh_3, RCOCl, DCE, rt (**38a**: 52%; **38b**: 65%); e) 10% aq. LiOH, THF/H_2O (**39a**: 80%); **39b**: 50%); f) i. (**25b – k, n**), $CuSO_4 \cdot 5H_2O$, sodium ascorbate, H_2O/^{t}BuOH 1:1, rt; ii. Dowex 50 X 8 Na^+ form (**9-20**: 16-80%). for R^1, R^2, R^3 and R^4 see Table.

Biological evaluation. To evaluate the binding properties of the sialosides the following formats were applied: a surface plasmon resonance (SPR) based assay (Biacore) and a hapten inhibition assay. The affinities of **1** and **2-20** were determined by the SPR based assay and in addition, the inhibitory potencies of **1** and **4** were determined by hapten inhibition assay (details see below).

For SPR assays, recombinant protein, consisting of the three *N*-terminal domains of MAG and the Fc part of human IgG (MAG_{d1-3}-Fc) was captured on polyclonal anti-Fc antibody, which was covalently bound to the surface. A reference cell, providing only antibody was used for compensation of unspecific binding to the matrix. The test compounds were dissolved in running buffer and injected over the surface. The obtained sensorgrams were fitted according to a 1:1 binding model. Concerns that our test compounds might form micelles in aqueous solution or show surface activity were vanished by determining the critical micelle concentration (CMC) with high-precision microbalances and wire probes.

CMC determination. The device is measuring the energy that needs to be expended to pull the wire probes out of the solution. This energy corresponds to the surface pressure (mN/m), which is inversely proportional to the surface tension. Due to the fact that the energy is decreasing with higher surface activity and stays constant as soon as micelles are being formed, the CMC and/or surface activity of a compound can be detected with a dilution series. In the case of compound **1** no activity up to 1 mM was observed.

The determination of the affinities (Table 1) revealed that *N*-alkylation of 5'-nitroindole with methyl (→**2**) or ethyl (→**3**) led to a slight decrease in affinity. Therefore we led the focus on the investigation of various substituents in 5'-position (→ **4**-**8**). We replaced the nitro group by electron with-drawing as well as electron-donating groups to elucidate whether this substituents influence the affinity.

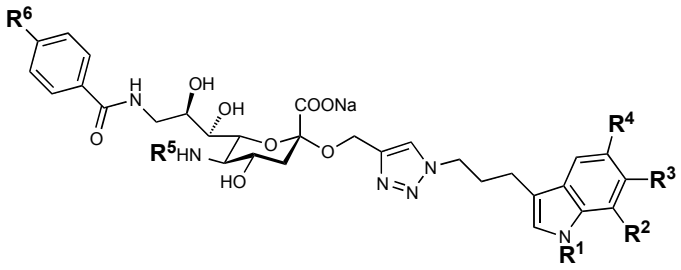
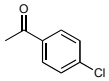


Table 1. Overview on affinities of antagonists **1 - 20,** determined by Biacore.

Compound	R^1	R^2	R^3	R^4	R^5	R^6	K_D [nM]
1	H	H	H	NO_2	Ac	H	190
2	Me	H	H	NO_2	Ac	H	445
3	Et	H	H	NO_2	Ac	H	333
4	H	H	H	H	Ac	H	167
5	H	H	H	OMe	Ac	H	322
9	H	H	H	CF_3	Ac	H	354
7	H	H		H	Ac	H	300
8	H	H		H	Ac	H	75
9	H	H	H	NO_2	FAc	H	50
10	H	H	H	NO_2	FAc	Cl	53
11	H	H	H	Cl	FAc	Cl	48
12	H	H	Cl	H	FAc	Cl	57
13	H	Cl	H	H	FAc	Cl	92
14	H	CH_3	H	H	FAc	Cl	96

15	H	H	H	F	FAc	Cl	67
16	H	H	H	OCH_3	FAc	Cl	79
17	H	H	H	$CH(CH_3)_2$	FAc	Cl	101
18	H	H	H	CN	FAc	Cl	66
19	H	H	H	SO_2Me	FAc	Cl	113
20	H	H	cyclopentane		FAc	Cl	98

The replacement of the nitro-group by trifluoromethyl (→**6**) or by the electron-donating methoxy-group (→**5**) yielded in a decreased affinity. In contrast, the unsubstituted indole (→**4**) showed a similar potency as the reference compound. These observations suggest on the one hand that the nitro-group might be interacting beneficially with the protein, whereas the other 5'-substituents are sterically too demanding. In the next step, we introduced substituents with increased lipophilic surface at 6'-position of the indole (→**7**, **8**). Unfortunately, cyclopropylacyl did not show any effect, however when 4-chlorobenzylacyl was introduced a gain in affinities by a factor of 3 was achieved. Here, the amelioration might result from additional favorable hydrophobic interactions. However, the compound was not soluble in aqueous buffer solution and therefore we returned our focus onto the initial lead **1.**

As mentioned above, we expected an improvement in binding affinity upon insertion of fluoroacetate in 5-position of **1**, and indeed this yielded an antagonist with a K_D of 50 nM (→**9**).

Molecular modeling studies. For an improved understanding of the biological data, compounds **4** and **9** and were docked to a homology model of MAG[13,14] and a molecular-dynamics simulation in aqueous solution was performed (4 ns at 300 K) using *Desmond*.[24]

In both antagonist-protein complexes, the sialic acid core established the crucial interactions responsible for recognition and binding *e.g.* the salt bridge between Arg118 and the carboxylate.[19] Moreover, hydrogen bonds between 5-NH and the carbonyl of Gln126, 8-OH and Thr128 and 9-NH and Tyr125 were formed in both cases.

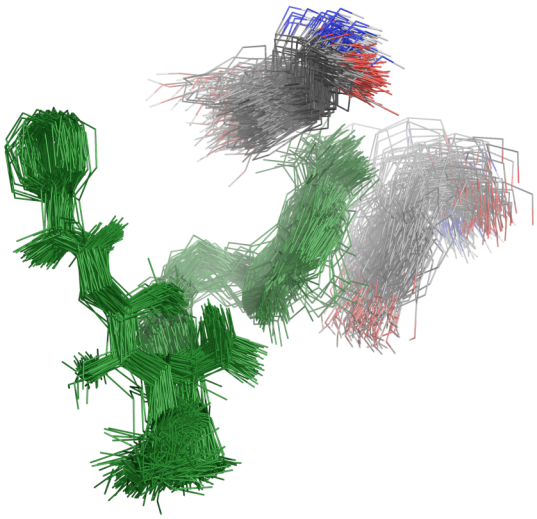
In the case of **9**, the 5'-nitroindole is embedded in a hydrophobic pocket lined by residues Tyr60, Tyr69 and Tyr116. Here, the indole-moiety is sandwiched by Tyr69 and 116, displaying an angle to Tyr69 of 17° and to Tyr116 an angle of 39° (Figure 2). It is

noteworthy that the π-π interactions are maintained during the whole simulation. Furthermore, the nitro-substituent interacts with Lys67 by dipolar interactions and seems to stabilize the position of the indole. The nitro-group is in the distance range of about 2.6 to 5.0 Å also found in other protein-ligand complexes.[25] In order to elucidate the role of the nitro-group experimentally, compounds **1** and **4** were tested with a MAG mutant K67A, where Lys67 is replaced, in a hapten inhibition assay as described below.

Simulations of ligand–protein complexes aim to understand details of the binding mode and to estimate the associated binding affinity. When comparing the binding of two similar ligands to a target protein, the difference in their free energy of binding (ΔΔG) can be calculated using free-energy perturbation (FEP).[26] Here, the calculation of the relative-free energy difference by using the free energy perturbation method (FEP, 1 ns) of the protein-ligand (ligand: **9**) complex and the complex with a virtual abolishment of the nitro-group in the ligand **9** led to a positive free energy difference, meaning that the abolishment of the nitro decreases the stability of the complex.

The linker contributes only marginally to the overall binding affinity but enables the alignment of the indole. Surprisingly, the linker seems to be in a rather locked conformation after binding (Figure 2, up). Finally, the FAc-substituent in 5-position of sialic acid contributes via hydrophobic interactions with Trp22.

a.



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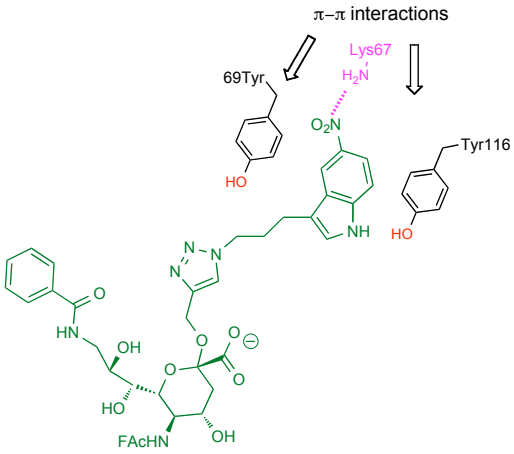

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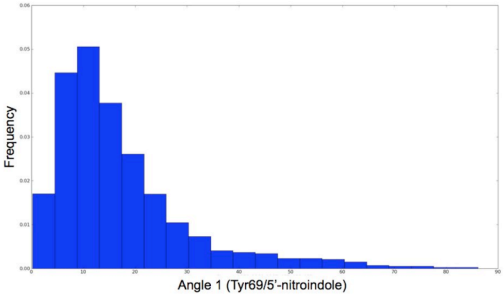


d.

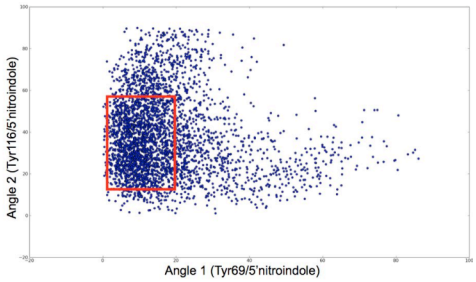
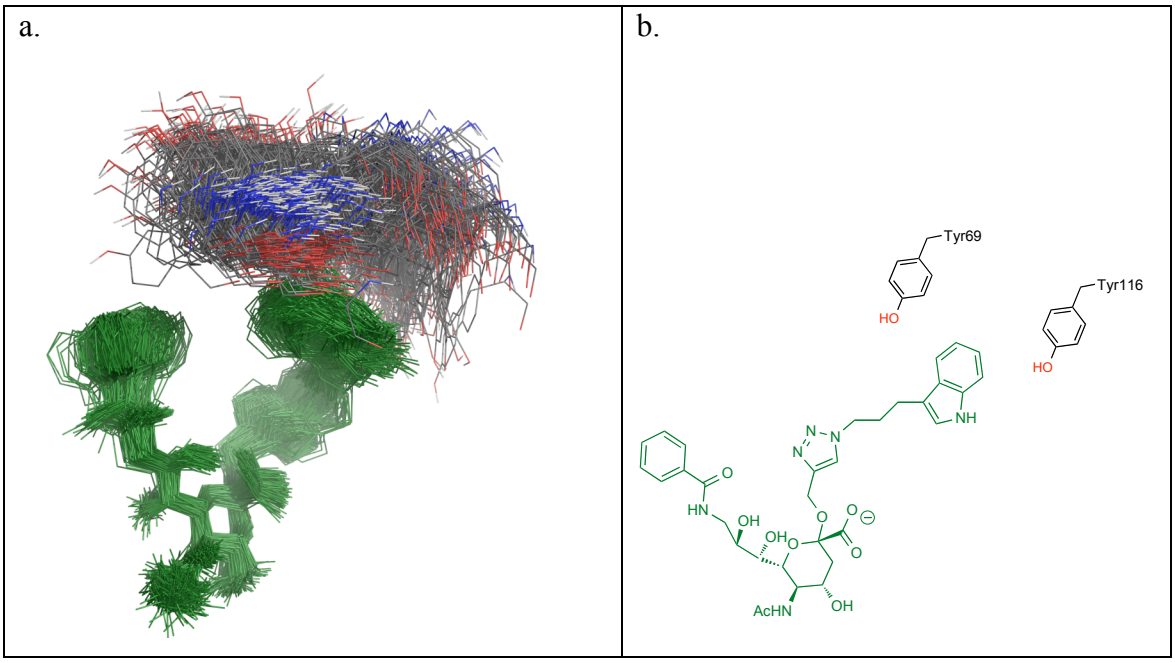


Figure 2. **a.** Conformations of antagonist **9** and the position of Tyr69 and Tyr116 during the dynamic simulation were clustered. 5'-Nitroindole interacts via π-π interactions with Tyr69 and Tyr116 resulting in a locked conformation of the linker. **b.** 2D-representation of **9** and its interactions with Tyr69 and 116. The dipolar interactions with Lys67 are depicted in pink. ***c.*** Plot of the frequency against the angle between Tyr69 and 5'-nitroindole. **d.** 2D-plot of angles between Tyr69/5'-nitroindole and Tyr116/5'-nitroindole shows that both tyrosine maintain a distinct angle during the simulation.

In the case of the unsubstituted indole no π-π interactions were observed. The angles of both tyrosines are around 70°-90° and the dispersion is clearly higher. Furthermore, the mobility of the linker was strikingly increased during the dynamic simulation (Figure 3). The nevertheless good binding affinity might be explained that no constraints raise from the linker positioning and that the core interactions might be slightly better established.



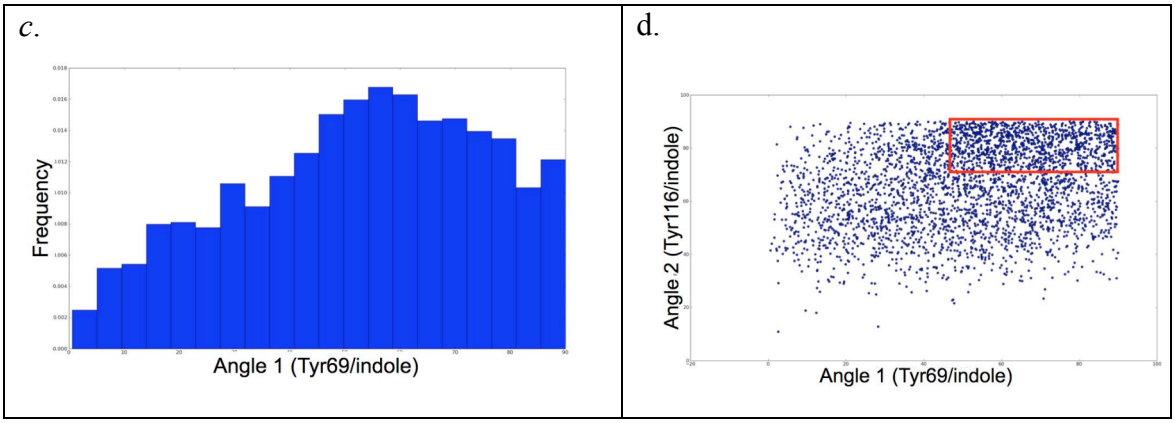


Figure 3. a. Conformations of antagonist **4** and position of Tyr69 and Tyr116 occurring during the dynamic simulation were clustered. Antagonist **4**, lacking the 5'-nitro substituent, seems not to be sandwiched and consequently shows increased mobility of the linker. **b.** 2D representation of **4**, Tyr69 and Tyr116. ***c.*** Plot of the frequency vs. the angle between Tyr69 and indole shows that no π-π interactions occur. **d.** 2D-plot of angles between Tyr69/indole and Tyr116/indole shows a higher dispersion and that the indole is almost perpendicular to both tyrosins.

In general, the findings discussed above are in agreement with the observation that replacement of nitro by other substituents decreases the affinity (→**6**), as no interaction with Lys67 is possible. Finally, modifications in 6'-position could be aligned in the hydrophobic cleft and additional hydrophobic interactions (→**8**) contribute to the binding affinity.

Hapten inhibition assay.

The molecular docking experiments of sialoside **9** described above suggested an interaction of the nitro residue of 5'-nitroindole with Lys67. To elucidate this interaction, two sialosides **1** and **4** were used in hapten inhibition assays with MAG_{d1-3}-Fc wt and a MAG mutant K67A. Using hapten inhibition assays, for each compound tested the concentrations required for 50 % inhibition (IC50) were determined in microtiter plates coated with fetuin as the binding target for MAG_{d1-3} Fc-chimeras[27]. Relative inhibitory potencies (rIP) were calculated from its IC50 ($IC50_{sample}$) and the IC50 of a reference compound ($IC50_{ref}$): $rIP = IC50_{ref}/IC50_{sample}$. At least three independent titrations were performed for each compound tested with seven or eight concentrations in triplicates. Relative inhibitory potencies (rIP) were calculated using compound **4** as reference (rIP of 1, Figure 5, Table 2).

Both substances lead to IC50-values in the low nanomolar range (Figure 4 a, Table 1). The presence of the nitro group in sialoside **1** leads to a 4.25-fold stronger inhibition compared to **4** for MAG_{d1-3}-Fc wt, whereas for MAG_{d1-3}-Fc K67A this increase is only 2.14-fold.

This supports the hypothesis that Lys67 contributes to binding via a hydrogen bond with the nitro group of compound **1**, as suggested in figure 4b. However, other interactions of the nitro group are likely to support binding to some extend, since its introduction also enhances inhibiton of the K67A mutant.

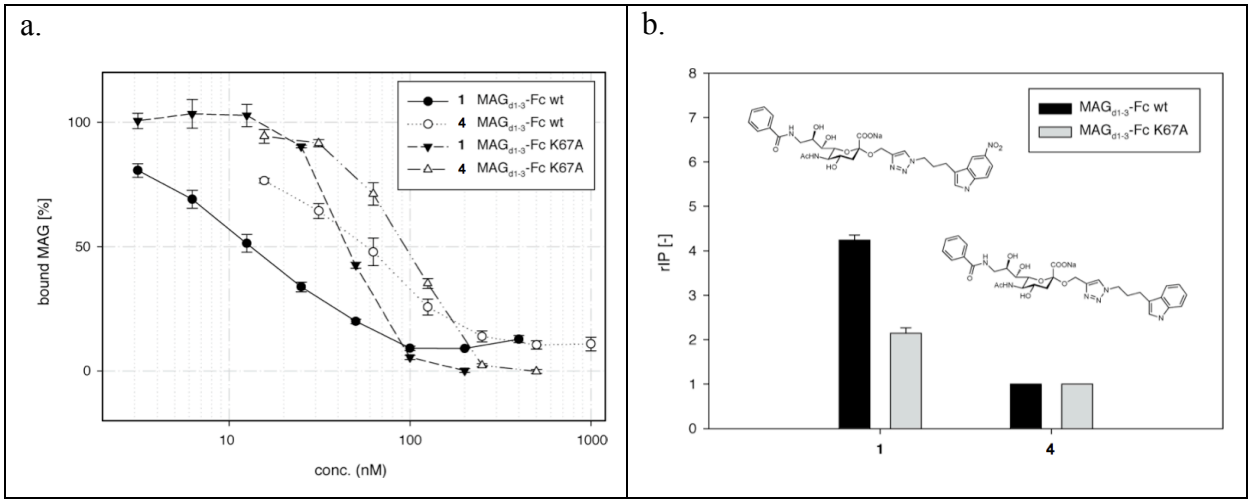


Figure 4. a. Inhibition curves of MAG_{d1-3}-Fc wild type (wt) and mutant K67A for the determination of IC50-values by hapten inhibition assay. **b.** rIP of sialosides **1** and **4** for both Fc-chimeras MAG wt and K67A. Sialoside **4** without nitro residue was calculated relative to sialoside **1** to obtain a factor of enhanced inhibition in consequence of the additional nitro group of **4**.

Table 2. Comparison of the half maximal inhibitory concentrations (IC50) and relative inhibitory potencies (rIP) for MAG_{d1-3}-Fc wild type (wt) and mutant K67A inhibited by sialosides **1** and **4** determined by hapten inhibition assay.

Compound	MAG_{d1-3}-Fc wt		MAG_{d1-3}-Fc K67A	
	IC50 [nM]	rIP [-]	IC50 [nM]	rIP [-]
1	13 ± 2	4.25 ± 0.19	41 ± 5	2.14 ± 0.21
4	53 ± 9	1.00 ± 0.00	88 ± 4	1.00 ± 0.00

Optimization with respect to medicinal chemistry aspects.

The new lead compound **4** showed improved binding affinity, however, there are two metabolic soft spots present in the molecule. At first we replaced the benzylamide by *p*-chlorobenzylamide, which showed an equivalent binding affinity (**→10**) and examined a series of ligands (**11**-**20**) where the nitro group was replaced by either halogen-, sulfonyl- or cynanide-substituents. Considering our findings with the MAG-mutant discussed above, we predicted interactions of the latter substituents with Lys67.

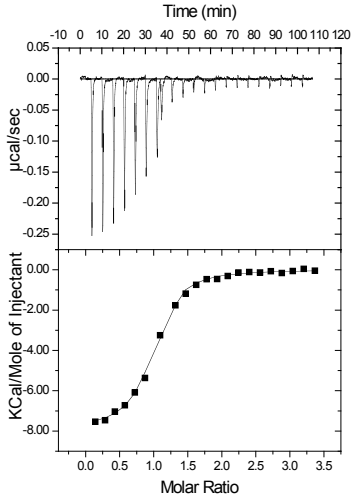
Antagonists **11** and **15** (Table 1) showed equivalent binding affinity of 48 nM and 57 nM respectively, and we demonstrated that the nitro group can be replaced by a chorine or

fluorine. In the case of **18** (5'-CN) we observed again a comparable binding affinity to **10**, unfortunately **19** (5'-SO_2Me) was less active. Furthermore, compound **12** with a chlorine substituent in the 6' position showed a comparable affinity. The introduction of substituents in 7'- position or larger substituents in 5'- position led to a decreased binding affinity.

Isothermal titration calorimetry (**ITC**).

Using our most potent compounds (**9-11**, **13**, **15** and **18**) and our initial lead **1**, we performed thermodynamic measurements in order to gain further insight into the different contributions to the overall binding affinity and determined the thermodynamic parameters ΔH, ΔS and ΔG.[28] For the experiments, MAG_{d1-3}-Fc was used. A solution of the ligands in HBS-E buffer was injected into a solution of MAG_{d1-3}-Fc (HBS-E buffer) at 25 °C. The experimental data were fitted to a theoretical titration curve (one site binding model) using *Origin version 7* software (MicroCal). The thermodynamic parameters ΔH, ΔS and ΔG were calculated according to equation **1** (Figure 5). The ITC experiments confirmed the high affinity of the antagonists, in the case of compounds **1** and **9** however, the obtained affinities were higher compared to the ones determined by Biacore. Nevertheless, we consider these results to be in good agreement as two different assays are compared and the trend is maintained.

Having now a closer look to the separate thermodynamic parameters, in all cases ligand binding is enthalpy driven.[29,30] As the enthalpy term highlights the difference of the ligands' interactions with the solvent compared to those with the protein,[31] this led to the conclusion that the antagonists exhibit directed interactions *e.g.* salt bridge, hydrogen bonds and van der Waals interactions and is in good agreement with our molecular modeling studies.



$$\Delta G = \Delta H - T\Delta S = - RT\ln K_D \quad (\mathbf{1})$$

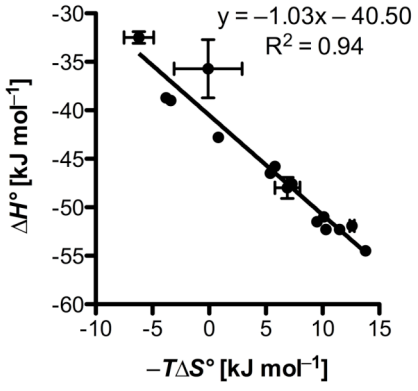


Figure 5. Enthalpogram of **9** (left). ΔG, ΔH and ΔS were calculated according to equation **1**, enthalpy-entropy compensation plot (right).

Table 3. Thermodynamic profile of selected MAG antagonists.*

	1	**9**	**10**	**11**	**12**	**15**	**18**
ΔG [kJ/mol]	-35.5	-39.2	-41.1	-42.5	-42.0	-42.4	-40.9
ΔH [kJ/mol]	-39.0	-32.1	-48.8	-38.8	-42.8	-39.0	-51.0
-TΔS [kJ/mol]	3.5	-7.1	7.7	-3.8	0.8	-3.4	10.1
K_D [nM]	588	136	64	35	44	37	69

*The stoichiometry N was in all measurements 1.01±0.03.

We observed, that the difference in the total binding energy results from the entropy term. Substitution by a halogen (→**11**, **15**) in 5'-position leads to an enhanced entropy, whereas the compounds with the 5'-nitro (→**10**) or 5'-cyanide (→**18**) suffer from a loss in entropy. We also observed this effect in the case of compound **19** (data not shown). This could be the result, at least partial, from the enforced orientation upon interaction with Lys 67, which is not as pronounced with the chlorine or fluorine substituents. An enthalpy-entropy plot revealed that we faced an enthalpy-entropy compensation effect (Figure 5), which has been already observed in the case of CD22.[33]

Conclusion. A small library of high affinity ligands was synthesized following medicinal chemistry approaches and investigated by Biacore and ITC. The effects of various modifications on the indole moiety were elucidated. Here, *N*-alkylation as well as substitution with sterically demanding substituents decreased binding slightly. Substitution with halogens or cyanide instead yielded an increase in binding affinity. Also the insertion of the 4-chlorobenzyl acyl in 6'-position yielded an improvement in affinity. With respect to the binding mode, 5'-nitro seems to be required for stabilizing the geometric orientation of the indole. Furthermore, we showed that the binding is enthalpy driven, however, the ligands entropy makes the difference with respect to the total binding affinity. Finally, compounds **11** and **15**, having a fluoroacetate substiuent at 5-position of neuraminic acid, a *p*-chlorobenzamide in 9-position and a chloro or fluoro substituent in 5'-position of the indole moiety were identified as the most potent antagonist for MAG to date, binding in the low nanomolar range.

Experimental part.

Chemistry. NMR spectra were recorded on a Bruker Avance DMX-500 (500 MHz) spectrometer. Assignment of ^{1}H and ^{13}C NMR spectra was achieved using 2D methods (COSY, HSQC, TOCSY). Chemical shifts are expressed in ppm using residual $CHCl_3$, CHD_2OD and HDO as references. Optical rotations were measured using Perkin-Elmer Polarimeters 241 and 341. MS analyses were carried out using a Waters Micromass ZQ Detector system. The spectra were recorded in positive or negative ESI mode. The HPLC/HRMS analyses were carried out using an Agilent 1100 equipped with a photodiode array detector and a Micromass QTOF I equipped with a 4 GHz digital-time converter. All target compounds exhibit a purity of ≥ 95%. Reactions were monitored by TLC using glass plates coated with silica gel 60 F_{254} (Merck) and visualized by using UV light and/or by charring with a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H_2SO_4). Column chromatography was performed on silica gel (Uetikon, 40-60 mesh). Methanol was dried by refluxing with sodium methoxide and distilled immediately before use. Pyridine was freshly distilled under argon over CaH_2. Dichloromethane (DCM), dichloroethane (DCE), acetonitrile (ACN), nitromethane, toluene, and benzene were dried by filtration over Al_2O_3 (Fluka, type 5016 A basic). Molecular sieves (3 Å) were activated under vacuum at 500 °C for 2 h immediately before use. If not stated otherwise, all starting materials were commercially available.

General procedure A: (23f – i)

To a solution of hydrazine-HCl (2.5 mmol) in aq. 4% H_2SO_4 (4 mL) and *N*,*N*-dimethylacetamide (DMAc, 10 mL), 3,4 dihydro-2*H*-pyran (2.5 mmol) was added dropwise. The reaction was aged for 2 h, then cooled to rt, extracted with ethylacetate (20 mL) and washed with water (3 x 20 mL). The aqueous layers were extracted with ethylacetate (20 mL) and the combined organic layers were dried over Na_2SO_4, filtered and evaporated. The crude product was purified by chromatography.

General procedure B: Appel-reaction (25a - o).

The alcohol (1.0 eq) was dissolved in dry MeCN and cooled to 0 °C. Afterwards, triphenylphosphine (1.2-1.5 eq) was added, followed by successive addition of CBr_4 (1.2-1.5 eq). The mixture was stirred at rt for 2 h and then diluted with ethyl acetate, washed with H_2O, brine and dried over Na_2SO_4. The solvent was removed under vacuum and the residue

was purified by chromatography (PE/ ethyl acetate) to yield the corresponding bromide. Afterwards, to a solution of bromide (1.0 eq) in dry DMF, NaN_3 (2.0-5.0 eq) was added at rt and stirring was continued overnight. Afterwards, the solvent was removed under vacuum and the residue was purified by chromatography (petroleum ether/ ethyl acetate) to afford the desired azides.

General procedure C: click-reaction (2 - 20).

To a mixture of acetylene (1.0 eq) in degassed $^{t}BuOH:H_2O$ (v/v, 1:1), azide (1.2-1.5 eq), $CuSO_4 \cdot 5H_2O$ (0.25 eq) and sodium ascorbate (0.5 eq) were added under argon atmosphere. The reaction mixture was stirred at rt overnight. Afterwards, the solvent was removed under reduced pressure and the residue was purified by reverse phase chromatography (Merck LiChroprep RP-18, 5% gradient of MeOH in water), Dowex 50x8 (Na^+ type) ion exchange chromatography and P2 size exclusion chromatography to afford the pure test compounds.

3-(6-chloro-1*H*-indol-3-yl)propan-1-ol (23f). Prepared from **22f** (400 mg, 2.234 mmol) according to general procedure A to afford **23f** (53 mg, 11%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.94 – 2.03 (m, 2H, CH_2), 3.01 – 3.09 (m, 2H, CH_2), 3.73 (t, *J* = 6.5 Hz, 2H, CH_2), 6.95 (s, 1H, CH_{ar}), 7.00 – 7.08 (m, 2H, CH_{ar}), 7.16 – 7.23 (m, 1H, CH_{ar}), 8.27 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.73, 34.61, 62.73 (CH_2), 110.14, 116.53, 120.45, 122.56, 123.12, 124.33, 126.57, 138.14 (C-Ar); ESI-MS *m/z* calcd for $C_{11}H_{12}ClNO$ $[M]^+$: 209.06; found: 209.89.

3-(5-isopropyl-1*H*-indol-3-yl)propan-1-ol (23g). Prepared from **22g** (300 mg, 1.6069 mmol) according to general procedure A to afford **23g** (152 mg, 43%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.34 (d, *J* = 6.9 Hz, 6H, 2 CH_3), 1.94 – 2.07 (m, 2H, CH_2), 2.88 (t, *J* = 7.5 Hz, 2H, CH_2), 3.05 (hept, *J* = 6.9 Hz, 1H, CH), 3.77 (t, *J* = 6.4 Hz, 2H, CH_2), 6.98 (s, 1H, CH_{ar}), 7.12 (dd, *J* = 1.4, 8.4 Hz, 1H, CH_{ar}), 7.30 (d, *J* = 8.4 Hz, 1H, CH_{ar}), 7.46 (s, 1H, CH_{ar}); ^{13}C NMR (126 MHz, $CDCl_3$) δ 21.58 (CH_2), 24.94 (2C, CH_3), 33.06 (CH_2), 34.49 (CH), 62.97 (CH_2), 111.09, 115.93, 115.95, 121.37, 121.63, 127.70, 135.18, 140.17 (C-Ar); ESI-MS *m/z* calcd for $C_{14}H_{19}NO$ $[M]^+$: 217.14; found: 217.94;

3-(5,6-cyclopropyl-1*H*-indol-3-yl)propan-1-ol (23h). Prepared from **22h** (200 mg, 1.0831 mmol) according to general procedure A to afford **23h** (140 mg, 60%).^{1}H NMR (500 MHz,

$CDCl_3$) δ 1.86 – 1.94 (m, 2H, CH_2), 2.04 (p, *J* = 7.3 Hz, 2H, CH_2^{cyc}), 2.74 (t, *J* = 7.4 Hz, 2H, CH_2), 2.90 (t, *J* = 7.3 Hz, 4H, 2 CH_2^{cyc}), 3.64 (t, *J* = 6.4 Hz, 2H, CH_2), 6.82 (d, *J* = 2.0 Hz, 1H, CH_{ar}), 7.09 (s, 1H, CH_{ar}), 7.34 (s, 1H, CH_{ar}), 7.70 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.73 (CH_2), 26.86, 32.57, 32.67 (CH_2^{cyc}), 33.05 (CH_2), 62.96 (CH_2), 106.68, 113.85, 118.87, 120.95, 126.82, 136.05, 136.08, 139.36 (C-Ar); ESI-MS *m/z* calcd for $C_{14}H_{17}NO$ $[M]^+$: 215.13; found: 215.92.

3-(7-chloro-1*H*-indol-3-yl)propan-1-ol (23i). Prepared from **22i** (300 mg, 1.6755 mmol) according to general procedure A to afford **23i** (80 mg, 22%).^{1}H NMR (500 MHz, $CDCl_3$) δ 1.91 – 2.02 (m, 2H, CH_2), 2.84 (t, *J* = 7.4 Hz, 2H, CH_2), 3.71 (t, *J* = 6.4 Hz, 2H, CH_2), 6.92 – 7.13 (m, 2H, CH_{ar}), 7.17 (d, *J* = 7.3 Hz, 1H, CH_{ar}), 7.50 (d, *J* = 7.9 Hz, 1H, CH_{ar}), 8.16 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 21.63, 33.12, 62.71 (CH_2), 116.83, 117.42, 117.78, 120.22, 121.55, 122.11, 129.20, 133.84 (C-Ar); ESI-MS *m/z* calcd for $C_{11}H_{12}ClNO$ $[M]^+$: 209.06; found: 209.85.

3-(3-Azidopropyl)-1*H*-indole (25a). Prepared from **23a** (494 mg, 2.82 mmol) according to general procedure B to afford **25a** (277 mg, two steps 50%) after chromatography as yellow oil. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.02 (m, 2H, CH_2), 2.88 (t, *J* = 7.5 Hz, 2H, CH_2), 3.34 (t, *J* = 7.0 Hz, 2H, CH_2), 7.01 (s, 1H, CH_{ar}), 7.15 (t, *J* = 7.0 Hz, 1H, CH_{ar}), 7.22 (t, *J* = 7.0 Hz, 1H, CH_{ar}), 7.37 (d, *J* = 8.0 Hz, 1H, CH_{ar}), 7.61 (d, *J* = 7.5 Hz, 1H, CH_{ar}), 7.99 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 22.1, 29.2, 50.9 (3 CH_2), 111.1, 115.0, 118.8, 119.3, 121.5, 122.1, 127.3, 136.3 (8 C-Ar); IR (film): ν 3415, 2929, 2097, 1456, 742 cm^{-1}.

3-(3-Azidopropyl)-5-methoxy-1*H*-indole (25b). Prepared from **23b** (523 mg, 2.548 mmol) according to general procedure B to afford **25b** (396 mg, two steps 68%) after chromatography as yellow oil. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.01 (m, 2H, CH_2), 2.85 (t, *J* = 7.0 Hz, 2H, CH_2), 3.36 (t, *J* = 7.0 Hz, 2H, CH_2), 3.89 (s, 3H, OMe), 6.88 (dd, *J* = 9.0, 2.5 Hz, 1H, CH_{ar}), 7.00 (d, *J* = 2.5 Hz, 1H, CH_{ar}), 7.05 (d, *J* = 2.0 Hz, 1H, CH_{ar}), 7.27 (d, *J* = 3.5 Hz, 1H, CH_{ar}), 7.87 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 22.1, 29.1, 50.8 (3 CH_2), 55.9 (OMe), 100.6, 111.8, 112.2, 114.7, 122.3, 127.7, 131.5, 153.9 (8 C-Ar); IR (film): ν 3415, 2938, 2097, 1485, 1214 cm^{-1}.

3-(3-Azidopropyl)-5-chloro-1*H*-indole (25c). Prepared from **23c** (99.4 mg, 0.4741 mmol) according to general procedure B to afford **25c** (86 mg, two steps 77%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.94 (p, *J* = 7.0 Hz, 2H, CH_2), 2.78 (t, *J* = 7.4 Hz, 2H, CH_2), 3.28 (t, *J* = 6.7 Hz, 2H, CH_2), 6.99 (d, *J* = 1.9 Hz, 1H, CH_{ar}), 7.12 (m, 1H, CH_{ar}), 7.22 (d, *J* = 3.8 Hz, 1H, CH_{ar}), 7.51 (d, *J* = 1.6 Hz, 1H, CH_{ar}), 7.95 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.20, 29.36, 51.03 (CH_2), 112.36, 115.13, 118.57, 122.62, 123.15, 125.36, 128.67, 134.92 (C-Ar); ESI-MS *m/z* calcd for $C_{11}H_{11}ClN_4$ $[M]^+$: 234.08; found: 234.82; IR (film): ν 3435, 2929, 2098, 1463 cm^{-1}.

3-(3-Azidopropyl)-5-fluoro-1*H*-indole (25d). Prepared from **23d** (65.7 mg, 0.3395 mmol) according to general procedure B to afford **25d** (54 mg, two steps 73%) after chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.83 – 1.94 (m, 2H, CH_2), 2.70 (t, *J* = 7.4 Hz, 2H, CH_2), 3.21 (t, *J* = 6.7 Hz, 2H, CH_2), 6.85 (td, $^{3}J_{1,2}$ = 2.5 Hz, $^{3}J_{H,F}$ 9.0 Hz, 1H, CH_{ar}), 6.92 (s, 1H, CH_{ar}), 7.14 (m, 2H, CH_{ar}), 7.85 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.20, 29.24, 50.98 (CH_2), 103.85 (d, $^{2}J_{C,F}$ = 23.29 Hz, C-4_{indole}), 110.57 (d, $^{2}J_{C,F}$ = 26.35, C-6_{indole}), 111.95 (d, $^{3}J_{C,F}$ = 9.68 Hz, C-7_{indole}), 115.36 (d, $^{4}J_{C,F}$ = 4.79 Hz, C-8_{indole}), 123.57 (indole), 127.86 (d, $^{3}J_{C,F}$ = 9.54 Hz, C-3_{indole}), 133.03 (indole), 157.88 (d, $^{1}J_{C,F}$ = 234.40 Hz, C-5_{indole}); ESI-MS *m/z* calcd for $C_{11}H_{11}FN_4$ $[M]^+$: 218.23; found: 218.88; IR (film): ν 3429, 2927, 2098, 1485 cm^{-1}.

3-(3-Azidopropyl)-7-methyl-1*H*-indole (25e). Prepared from **23e** (82 mg, 0.4333 mmol) according to general procedure B to afford **25e** (64 mg, two steps 70%) after chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.96 – 2.08 (m, 2H, CH_2), 2.49 (s, 3H, CH_3), 2.88 (t, *J* = 7.3 Hz, 2H, CH_2), 3.33 (t, *J* = 6.7 Hz, 2H, CH_2), 6.97 – 7.05 (m, 2H, CH_{ar}), 7.08 (td, *J* = 2.0, 7.4 Hz, 1H, CH_{ar}), 7.47 (d, *J* = 7.8 Hz, 1H, CH_{ar}), 7.86 (s, 1H, NH); **^{13}C** NMR (126 MHz, $CDCl_3$) δ 16.74 (CH_3), 22.44, 29.45, 51.09 (CH_2), 115.72, 116.72, 119.77, 120.54, 121.49, 122.79, 127.04, 136.16 (C-Ar); ESI-MS *m/z* calcd for $C_{12}H_{14}N_4$ $[M]^+$: 214.12; found: 214.88; IR (film): ν 3418, 2930, 2097 cm^{-1}.

3-(3-Azidopropyl)-6-chloro-1*H*-indole (25f). Prepared from **23f** (43 mg, 0.2051 mmol) according to general procedure B to afford **25f** (35 mg, two steps 75%) after chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.00 – 2.14 (m, 2H, CH_2), 3.10 (t, *J* = 7.5 Hz, 2H, CH_2), 3.37 (t, *J* = 6.8 Hz, 2H, CH_2), 7.03 (d, *J* = 1.9 Hz, 1H, CH_{ar}), 7.09 – 7.15 (m, 2H, CH_{ar}), 7.24 –

7.31 (m, 1H, CH_{ar}), 8.07 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 23.53, 30.86, 51.06 (CH_2), 110.13, 115.82, 120.66, 122.80, 123.31, 124.27, 126.56, 138.11 (C-Ar); ESI-MS *m/z* calcd for $C_{11}H_{11}ClN_4$ $[M]^+$: 234.08; found: 234.81; IR (film): ν 3429, 2929, 2097, 1437 cm^{-1}.

3-(3-Azidopropyl)-5-isopropyl-1*H*-indole (25g). Prepared from **23g** (152 mg, 0.6995 mmol) according to general procedure B to afford **25g** (144 mg, two steps 85%) after chromatography. ^1H NMR (500 MHz, $CDCl_3$) δ 1.32 (d, *J* = 6.9 Hz, 6H, CH_3), 2.01 (p, *J* = 6.9 Hz, 2H, CH_2), 2.86 (t, *J* = 7.3 Hz, 2H, CH_2), 3.03 (hept, *J* = 6.9 Hz, 1H, CH), 3.34 (t, *J* = 6.8 Hz, 2H, CH_2), 6.95 (s, 1H, CH_{ar}), 7.11 (dd, *J* = 1.5, 8.4 Hz, 1H, CH_{ar}), 7.28 (d, *J* = 8.4 Hz, 1H, CH_{ar}), 7.42 (s, 1H, Indole), 7.83 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.31 (CH_2), 24.92 (2C, CH_3), 29.41 (CH_2), 34.49 (CH), 51.14 (CH_2), 111.14, 115.03, 115.87, 121.47, 121.91, 127.57, 135.17, 140.31 (C-Ar); ESI-MS *m/z* calcd for $C_{14}H_{18}N_4$ $[M]^+$: 242.15; found: 242.07; IR (film): ν 3412, 2957, 2096 cm^{-1}.

3-(3-Azidopropyl)-5,6-cyclopropyl-1*H*-indole (25h). Prepared from **23h** (36 mg, 0.1672 mmol) according to general procedure B to afford **25h** (10 mg, two steps 25%) after chromatography. ^1H NMR (500 MHz, $CDCl_3$) δ 1.98 (p, *J* = 7.0 Hz, 2H, CH_2), 2.12 (dq, *J* = 7.3, 14.6 Hz, 2H, CH_2^{cyc}), 2.82 (t, *J* = 7.3 Hz, 2H, CH_2), 2.98 (td, *J* = 2.1, 7.2 Hz, 4H, 2 CH_2^{cyc}), 3.31 (t, *J* = 6.8 Hz, 2H, CH_2), 6.90 (d, *J* = 2.1 Hz, 1H, CH_{ar}), 7.18 (s, 1H, CH_{ar}), 7.40 (s, 1H, CH_{ar}), 7.75 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.44 (CH_2), 26.86 (CH_2^{cyc}), 29.44 (CH_2), 32.67, 33.05 (CH_2^{cyc}), 51.12 (CH_2), 106.73, 113.76, 114.70, 121.25, 126.67, 136.19, 136.25, 139.48 (C-Ar); ESI-MS *m/z* calcd for $C_{14}H_{16}N_4$ $[M]^+$: 240.13; found: 240.96; IR (film): ν 3401, 2934, 2094, 1646 cm^{-1}.

3-(3-Azidopropyl)-7-chloro-1*H*-indole (25i). Prepared from **23i** (80 mg, 0.3816 mmol) according to general procedure B to afford **25i** (10 mg, two steps 16%) after chromatography. ^1H NMR (500 MHz, $CDCl_3$) δ 1.91 – 2.02 (m, 2H, CH_2), 2.84 (t, *J* = 7.4 Hz, 2H, CH_2), 3.31 (t, *J* = 6.7 Hz, 2H, CH_2), 7.04 (t, *J* = 7.8 Hz, 2H, CH_{ar}), 7.18 (d, *J* = 7.6 Hz, 1H, CH_{ar}), 7.48 (d, *J* = 7.9 Hz, 1H, CH_{ar}), 8.17 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.40, 29.44, 51.01 (CH_2), 116.46, 116.91, 117.67, 120.36, 121.66, 122.38, 129.02, 133.85 (C-Ar); ESI-MS *m/z* calcd for $C_{11}H_{12}ClN_4$ $[M+H]^+$: 235.08; found: 235.82; IR (film): ν 3427, 2925, 2098, 1437 cm^{-1}.

3-(5-cyano-1*H*-indol-3-yl)propyl acetate (**26j**)**.** To a mixture of 3,4 dihydro-2*H*-pyran (242 µL, 2.65 mmol) in AcOH (6 mL) and HCl conc. (2 mL) at rt **22j** (150 mg, 0.8833 mmol) was added and the reaction mixture was stirred for 3 h at 100°C. After cooling to rt the reaction mixture was diluted in ethyl acetate (50 mL) and washed with water (40 mL) and brine (40 mL). The aqueous layers were extracted with ethyl acetate (40 mL) and the combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. Chromatography (petroleum ether/ ethyl acetate 2:1) of the residue gave pure **26j** (66 mg, 32%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.97 – 2.10 (m, 2H, CH_2), 2.06 (s, 3H, CH_3), 2.82 (t, *J* = 7.4 Hz, 2H, CH_2), 4.11 (t, *J* = 6.5 Hz, 2H, CH_2), 7.11 (m, 1H, CH_{ar}), 7.39 (s, 2H, CH_{ar}), 7.92 (s, 1H, CH_{ar}), 8.40 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 21.14, 21.17 (CH_2, CH_3), 44.34 (CH_2), 63.92 (CH_2), 102.54, 112.22, 116.56, 121.00, 123,73, 124.73, 125.12, 127.48, 138.21 (C-Ar, CN) 171.39 (C=O); ESI-MS *m/z* calcd for $C_{14}H_{14}NaN_2O_2$ $[M+Na]^+$: 265.09; found: 264.92; IR (film): ν 3343, 2924, 2219, 1734, 1246 cm^{-1}.

3-(5-methylsulfonyl-1*H*-indol-3-yl)propyl acetate (26k). Prepared from **22k** (150 mg, 0.5926 mmol) according to procedure for compound 26j to afford **26k** (65 mg, 37%). ^{1}H NMR (500 MHz, CD_3OD) δ 2.00 – 2.09 (m, 2H, CH_2), 2.05 (s, 3H, CH_3), 2.90 (t, *J* = 7.4 Hz, 2H, CH_2), 3.11 (s, 3H, CH_3), 4.10 (t, *J* = 6.4 Hz, 2H, CH_2), 7.27 (s, 1H, CH_{ar}), 7.54 (d, *J* = 8.5 Hz, 1H, CH_{ar}), 7.65 (dd, *J* = 1.8, 8.6 Hz, 1H, CH_{ar}), 8.17 (d, *J* = 1.5 Hz, 1H, CH_{ar}). ^{13}C NMR (126 MHz, CD_3OD) δ 21.00 (CH_3), 22.14, 30.66 (CH_2), 45.46 (CH_3), 65.17 (CH_2), 113.19, 117.57, 120.36, 120.83, 126.31, 128.60, 131.77, 140.62 (C-Ar), 173.31 (C=O). ESI-MS *m/z* calcd for $C_{14}H_{17}NaNO_4S$ $[M+Na]^+$: 318.08; found: 318.02.

3-(5-cyano-1*H*-indol-3-yl)propan-1-ol (23j). To a mixture of **26j** (80 mg, 0.3302 mmol) in MeOH (2 mL) an equivalent freshly prepared NaOMe was added. After 2 h the reaction mixture was neutralized with AcOH and evaporated to dryness. Chromatography (petroleum ether/ethyl acetate 3:2) of the residue gave **23j** (18 mg, 27%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.92 – 1.99 (m, 2H, CH_2), 2.84 (t, *J* = 7.5 Hz, 2H, CH_2), 3.71 (t, *J* = 6.3 Hz, 2H, CH_2), 7.10 (s, 1H, CH_{ar}), 7.38 (d, *J* = 0.8 Hz, 2H, CH_{ar}), 7.93 (s, 1H, CH_{ar}), 8.49 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 21.22, 33.01, 62.45 (CH_2), 102.39, 112.19, 117.24, 121.11, 123.64, 124.89, 125.07, 127.62, 138.22 (CN, C-Ar); ESI-MS *m/z* calcd for $C_{12}H_{12}NaN_2O$ $[M+Na]^+$: 223.08; found: 222.81; IR (film): ν 3339, 2932, 2220, 1472, 1056 cm^{-1}.

3-(5-methylsulfonyl-1*H*-indol-3-yl)propan-1-ol (23k). Prepared from **26k** (102 mg, 0.3454 mmol) according to procedure for compound 23j to afford **23k** (37 mg, 42%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.90 – 2.01 (m, 2H, CH_2), 2.88 (t, *J* = 7.5 Hz, 2H, CH_2), 3.07 (s, 3H, CH_3), 3.72 (t, *J* = 6.3 Hz, 2H, CH_2), 7.15 (s, 1H, CH_{ar}), 7.46 (d, *J* = 8.5 Hz, 1H, CH_{ar}), 7.70 (dd, *J* = 1.7, 8.5 Hz, 1H, CH_{ar}), 8.24 (d, *J* = 1.6 Hz, 1H, CH_{ar}), 8.35 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 21.05, 32.92 (CH_2), 45.24 (CH_3), 61.78 (CH_2), 112.06, 117.26, 119.61, 119.74, 124.47, 127.24, 130.08, 138.97 (C-Ar); ESI-MS *m/z* calcd for $C_{12}H_{15}NaNO_3S$ $[M+Na]^+$: 276.06; found: 275.93.

3-(3-Azidopropyl)-5-cyano-1*H*-indole (25j). Prepared from **23j** (30 mg, 0.1498 mmol) according to general procedure B to afford **25j** (25 mg, two steps 70%) after chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.90 – 2.01 (m, 2H, CH_2), 2.84 (t, *J* = 7.4 Hz, 2H, CH_2), 3.32 (t, *J* = 6.7 Hz, 2H, CH_2), 7.12 (d, *J* = 2.0 Hz, 1H, CH_{ar}), 7.33 – 7.56 (m, 2H, CH_{ar}), 7.92 (s, 1H, CH_{ar}), 8.47 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.00, 29.34, 50.92 (CH_2), 102.51, 112.30, 116.21, 121.03, 123.93, 124.72, 125.16, 127.42, 138.23 (CN, C-Ar); ESI-MS *m/z* calcd for $C_{12}H_{11}NaN_5$ $[M+Na]^+$: 248.09; found: 247.89; IR (film): ν 3333, 2926, 2217, 2093 cm^{-1}.

3-(3-Azidopropyl)-5-methylsulfonyl-1*H*-indole (25k). Prepared from **23k** (37 mg, 0.1462 mmol) according to general procedure B to afford **25k** (22 mg, two steps 55%) after chromatography. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.91 – 2.02 (m, 2H, CH_2), 2.85 (t, *J* = 7.4 Hz, 2H, CH_2), 3.08 (s, 3H, CH_3), 3.30 (t, *J* = 6.7 Hz, 2H, CH_2), 7.14 (d, *J* = 2.1 Hz, 1H, Indole), 7.46 (d, *J* = 8.6 Hz, 1H, CH_{ar}), 7.68 (dd, *J* = 1.7, 8.6 Hz, 1H, CH_{ar}), 8.20 (d, *J* = 1.5 Hz, 1H, CH_{ar}), 8.68 (s, 1H, NH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 22.02, 29.36 (CH_2), 45.47 (CH_3), 50.93 (CH_2), 112.21, 116.89, 119.82, 120.57, 124.43, 127.25, 131.30, 138.89 (C-Ar); ESI-MS *m/z* calcd for $C_{12}H_{14}NaN_4O_2S$ $[M+Na]^+$: 301.07; found: 300.99; IR (film): ν 3351, 2925, 2098, 1293, 1142 cm^{-1}.

3-(1*H*-indol-3-yl)propyl acetate (27). To a solution of **23a** (3.90 g, 22.3 mmol) and DMAP (133 mg, 1.1 mmol) in pyridine (30 mL), Ac_2O (20 mL) was added and the reaction mixture was stirred at rt overnight. The solvent was co-evaporated with toluene and the residue purified by chromatography (petroleum ether/ethyl acetate, 6:1 to 4:1) to yield **27** (4.44 g, 92%) as a colourless solid. Analytic data were in accordance with published data.

3-(1-Tosyl-1*H*-indol-3-yl)propyl acetate (28). To an ice-cold slurry of NaH (60%, 1.31 g, 32.7 mmol) in THF (5 mL) a solution of **27** (2.34 g, 10.8 mmol) in THF (15 mL) was added. After stirring for 1 h at 0 °C, tosyl chloride (6.15 g, 32.3 mmol) was added over a period of 1 h in three portions and the reaction mixture was stirred for another 2 h at 0 °C. After quenching by adding aq. NH_4Cl (10 mL) the mixture was transferred into a separation funnel, diluted with ethyl acetate (100 mL) and washed with aq. NH_4Cl (100 mL) and water (100 mL). The aqueous layers were extracted with ethyl acetate (100 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by chromatography (toluene/ethyl acetate, 1:0 to 2:1) to yield **28** (2.81 g, 70 %) as colourless oil. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.01 (m, 2H, CH_2), 2.05 (s, 3H, OAc), 2.33 (s, 3H, CH_3), 2.74 (t, J = 7.5 Hz, 2H, CH_2), 4.10 (t, J = 6.4 Hz, 2H, CH_2), 7.20 - 7.26 (m, 3H, CH_{ar}), 7.29-7.34 (m, 2H, CH_{ar}), 7.46 (m, 1H, CH_{ar}), 7.74 (m, 1H, CH_{ar}), 7.98 (m, 1H, CH_{ar}); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 20.9 (OAc), 21.4 (CH_2), 21.5 (CH_3), 27.9 (CH_2), 63.7 (CH_2), 113.8, 119.3, 122.0 (C-3), 122.9 (C-2), 123.0, 124.6, 126.7, 129.8, 130.8, 135.3, 135.4, 144.7 (C-Ar), 171.1 (CO).

3-((6-Cyclopropanecarbonyl)-1*H*-indol-3-yl)propan-1-ol (23l). To an ice-cold solution of **28** (302 mg, 0.80 mmol) in nitromethane (6 mL), cyclopropanecarbonyl chloride (340 µL, 3.7 mmol) and $AlCl_3$ (545 mg, 4.1 mmol) were added. After stirring for 1.5 h at 0 °C, the reaction mixture was quenched by addition of water (10 mL). The mixture was transferred into a separation funnel, diluted with ethyl acetate (40 mL) and subsequently washed with water (40 mL) and brine (40 mL). The aqueous layers were extracted with ethyl acetate (40 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was redissolved in methanol (5 mL) and treated with aqueous solution of NaOH (6 M, 5 mL). After refluxing for 1 h, the mixture was transferred into a separation funnel with CH_2Cl_2 (40 mL), neutralized with aq. HCl and extracted with water (2 × 40 mL). The organic layers were extracted with CH_2Cl_2 (2 × 40 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by chromatography (toluene/ethyl acetate, 2:1 to 1:2) to yield a inseparable mixture of 3-((5-cyclopropanecarbonyl)-1*H*-indol-3-yl)propan-1-ol and **23l** (121.2 mg, 61%) as a white solid. The mixture was reacted further and separation was accomplished in the next step.

3-((6-(4-Chlorophenylcarbonyl))-1*H*-indol-3-yl)propan-1-ol (23m). To an ice-cold solution of **28** (408 mg, 1.10 mmol) in nitromethane (12 mL), 4-chlorobenzoyl chloride (560 µL, 4.40 mmol) and $AlCl_3$ (732 mg, 5.50 mmol) were added. After stirring for 2 h at 0 °C, the reaction mixture was quenched by addition of water (10 mL). The mixture was transferred into a separation funnel, diluted with ethyl acetate (40 mL) and subsequently washed with water (40 mL) and brine (40 mL). The aqueous layers were extracted with ethyl acetate (40 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was redissolved in methanol (8 mL) and treated with aq. NaOH (6 M, 8 mL). After stirring for 1.5 h at rt, the mixture was neutralized with aq. HCl, transferred into a separation funnel with CH_2Cl_2 (40 mL), and extracted with water (2 × 40 mL). The organic layers were extracted with CH_2Cl_2 (40 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified by chromatography (toluene/CH_2Cl_2/*i*-propanol, 25:5:1 to 4:5:1) to yield a inseparable mixture of 3-((5-(4-chlorophenylcarbonyl))-1*H*-indol-3-yl)propan-1-ol and **23m** (163 mg, 47%). The mixture was reacted further and separation was accomplished in the next step.

3-(3-Azidopropyl)-6-cyclopropanecarbonyl-1*H*-indole (25l). The mixture of **23l** and 3-((5-cyclopropanecarbonyl)-1*H*-indol-3-yl)propan-1-ol (112.0 mg, 0.460 mmol), CBr_4 (231 mg, 0.70 mmol) and triphenylphosphine (145 mg, 0.55 mmol) in dry DMF (1 mL) was shaking in an eppendorf tube (2 mL) at 50 °C and 900 rpm for 30 min. NaN_3 (60.0 mg, 0.923 mmol) was added and the reaction mixture was allowed to be shaken overnight at 50 °C. The reaction was quenched by addition of a few drops of water and directly purified by LC-MS to yield **25l** (44.4 mg, 36%) and 3-(3-azidopropyl)-5-cyclopropanecarbonyl-1*H*-indole (15 mg, 12%) as colourless solids. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.93 – 1.10 (m, 2H, CH_2) 1.21 – 1.32 (m, 2H, CH_2), 2.01 (p, *J* = 7.0 Hz, 2H, CH_2), 2.72 – 2.80 (m, 1H, CH), 2.89 (t, *J* = 7.4 Hz, 2H, CH_2), 3.34 (t, *J* = 6.7 Hz, 2H, CH_2), 7.20 (d, *J* = 1.9 Hz, 1H, CH_{ar}), 7.65 (d, *J* = 8.4 Hz, 1H CH_{ar}), 7.75 – 7.90 (m, 1H CH_{ar}), 8.10 (s, 1H, CH_{ar}), 8.25 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.3 (CH_2), 17.1 (CH), 22.0, 29.3, 50.8 (3 CH_2), 111.9, 115.5, 118.4, 119.5, 125.3, 130.8, 132.4, 135.9 (8 C-Ar), 200.6 (CO).

3-(3-Azidopropyl)-6-(4-chlorophenylcarbonyl)-1*H*-indole (25m). The mixture of **23m** and 3-((5-(4-chlorophenylcarbonyl))-1*H*-indol-3-yl)propan-1-ol (104 mg, 0.33 mmol), CBr_4 (165

mg, 0.50 mmol) and triphenylphosphine (106 mg, 0.40 mmol) in dry DMF (1 mL) was shaken in an eppendorf tube (2 mL) at 50 °C and 900 rpm for 15 min. NaN_3 (44 mg, 0.68 mmol) was added and the reaction mixture was allowed to be shaken overnight at 50 °C. The reaction was quenched by addition of a few drops of water and directly purified by LC-MS to yield 3-(3-azidopropyl)-6-(4-chlorophenylcarbonyl)-1*H*-indole (4.5 mg, 4%) and **25m** (42 mg, 37%) as yellow solids.

3-(3-((^{t}Butyldimethylsilyl)oxy)propyl-5-trifluoromethyl-2-trimethylsilyl-1*H*-indole (31o). A mixture of 29o (1.2 g, 4.2 mmol), 30[17] (2.3 g, 8.4 mmol), potassium acetate (2.1 g, 21.0 mmol), lithium chloride (178 mg, 4.2 mmol) and palladium(II) acetate (77 mg, 0.42 mmol) in dry DMF (10 mL) was heated at 70 - 75 °C under argon for 2.5 h. After cooling to rt, the reaction mixture was diluted with ether and ice-water, the aqueous layer was separated and extracted with ethyl acetate (50 mL). The combined organic layers were washed with water (50 mL) and brine (50 mL), dried over Na_2SO_4 and evaporated. The residue was purified by chromatography (petroleum ether/ethyl acetate 8:1) to give 31o (1.7 g, 89%) after chromatography as a yellow oil.[1] H NMR ($CDCl_3$, 500 MHz): δ 0.12 (s, 6H, $2CH_3$), 0.43 (s, 9H, $3CH_3$), 0.97 (s, 9H, $C(CH_3)_3$), 1.89 (m, 2H, CH_2), 2.95 (m, 2H, CH_2), 3.77 (t, *J* = 6.0 Hz, 2H, CH_2), 7.42 (m, 2H, CH_{ar}), 7.92 (s, 1H, CH_{ar}), 8.06 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ -5.3 (CH_3), -0.7 (CH_3), 18.4 ($C(CH_3)$), 22.4 (CH_2)?, 35.2 (), 62.9 (CH_2), 107.1, 111.0, 116.7 (q, *J* = 4.25 Hz), 118.9 (q, *J* = 3.25 Hz), 126.4, 128.2, 135.1, 139.4.

3-(5-Trifluoromethyl-1*H*-indol-3-yl)propan-1-ol (23o). To a solution of **31o** (1.7 g, 4.0 mmol) in MeCN (20 mL) was added sequentially 48% HF (2 mL) and stirred at rt. for 48 h. The mixture was then cautiously basified with satd aq. Na_2CO_3 and extracted with ethyl acetate. The organic extract was washed with brine, dried over Na_2SO_4 and evaporated to give a viscous solid. Purification by chromatography (petroleum ether/ ethyl acetate 1:1) gave **23o** (671 mg, 65%) after chromatography as yellow oil.[1] H NMR ($CDCl_3$, 500 MHz): δ 2.01 (m, 2H, CH_2), 2.89 (t, *J* = 7.5 Hz, 2H, CH_2), 3.75 (t, *J* = 6.5 Hz, 2H, CH_2), 7.11 (t, *J* = 1.0 Hz, 1H, CH_{ar}), 7.42 (d, *J* = 1.5 Hz, 2H, CH_{ar}), 7.90 (s, 1H, CH_{ar}), 8.17 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 21.1, 32.8, 62.4 (3 CH_2), 111.3, 116.6 (q, *J* = 4.3 Hz), 117.1, 118.8 (q, *J* = 3.5 Hz), 121.7 (q, *J* = 31.8 Hz), 122.9, 126.5, 126.9, 137.6.

3-(3-Azidopropyl)-5-trifluoromethyl-1*H*-indole (25o). Prepared from **23o** (607 mg, 2.50 mmol) according to general procedure B to afford **25o** (533 mg, two steps 80%) after chromatography as yellow oil. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.02 (m, 2H, CH_2), 2.90 (t, *J* = 7.5 Hz, 2H, CH_2), 3.36 (t, *J* = 6.5 Hz, 2H, CH_2), 7.12 (d, *J* = 2.0 Hz, 1H, CH_{ar}), 7.44 (m, 2H, CH_{ar}), 7.89 (s, 1H, CH_{ar}), 8.17 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 21.9, 29.1, 50.8 (3 CH_2), 111.4, 116.1, 116.5 (q, *J* = 4.3 Hz), 118.9 (q, *J* = 3.5 Hz), 121.9 (q, *J* = 31.5 Hz), 123.1, 124.3, 126.7, 137.6. IR (film): ν 3440, 2940, 2101, 1432, 1329, 1111 cm^{-1}.

3-(3-Azidopropyl)-1-methyl-5-nitro-1*H*-indole (32). To a stirred solution of **25n**[17] (48.5 mg, 198 μmol) in DMF (2 mL), powdered KOH (112 mg, 2.00 mmol) was added. Iodomethane (125 μL, 2.00 mmol) was added drop-wise and the reaction was stirred at rt for 2 h. The reaction mixture was quenched by addition of water (2 mL), transferred into a separation funnel with CH_2Cl_2 (20 mL) and extracted with water (2 × 20 mL). The aqueous layers were extracted with CH_2Cl_2 (20 mL). The combined organic layers were dried over Na_2SO_4, filtered and co-evaporated with toluene to dryness. The residue was purified by chromatography (petroleum ether/ethyl acetate, 9:1 to 1:1) to yield **32** (48 mg, 94%) as an yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.87 – 2.08 (m, 2H, CH_2), 2.88 (t, *J* = 7.4 Hz, 2H, CH_2), 3.35 (t, *J* = 6.7 Hz, 2H, CH_2), 3.81 (s, 3H, CH_3), 7.01 (s, 1H, CH_{ar}), 7.31 (t, *J* = 9.5 Hz, 1H, CH_{ar}), 8.13 (dd, *J* = 2.2, 9.0 Hz, 1H, CH_{ar}), 8.55 (d, *J* = 2.1 Hz, 1H, CH_{ar}). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.8, 29.4, 33.2 (3 CH_2), 50.7 (CH_3), 109.2, 116.4, 117.5, 129.5 (8C, C-Ar).

3-(3-Azidopropyl)-1-ethyl-5-nitro-1*H*-indole (33). To a stirred solution of **25n** (56.0 mg, 0.23 mmol) in DMF (2 mL), powdered KOH (129 mg, 2.30 mmol) was added. Iodoethane (185 μL, 2.30 mmol) was added drop-wise and the reaction mixture was stirred at rt for 2 h. The reaction was quenched by addition of water (2 mL), transferred into a separation funnel with CH_2Cl_2 (20 mL) and extracted with water (2 × 20 mL). The aqueous layers were extracted with CH_2Cl_2 (20 mL). The combined organic layers were dried over Na_2SO_4, filtered and co-evaporated with toluene to dryness. The residue was purified by chromatography (petroleum ether/ethyl acetate, 1:0 to 3:1) to yield **33** (57 mg, 92%) as an yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.48 (t, *J* = 7.3 Hz, 3H, CH_3), 1.96 – 2.04 (m, 2H, CH_2), 2.88 (t, *J* = 7.5 Hz, 2H, CH_2), 3.35 (t, *J* = 6.7 Hz, 2H, CH_2), 4.18 (q, *J* = 7.3 Hz, 2H, CH_2), 7.07 (s, 1H, CH_{ar}), 7.32 (d, *J* = 9.1 Hz, 1H, CH_{ar}), 8.11 (dd, *J* = 2.2, 9.1 Hz, 1H, CH_{ar}),

8.54 (d, J = 2.1 Hz, 1H, CH_{ar}); ^{13}C NMR (125 MHz, $CDCl_3$): δ 15.4 (CH_3), 21.9, 29.4, 41.4, 50.8 (4 CH_2), 109.1, 116.5, 117.3, 127.7 (8C, C-Ar).

Methyl [2-propynyl 4,7,8-tri-*O*-acetyl-9-azido-5-*t*butyrylcarbamat-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosid]onate (35). Compound **34**[17] (39.0 mg, 80 µmol) was reacted with Boc-anhydride (33 mg, 0.15 mmol) and DMAP (2.5 mg, 20 µmol) at 50 °C for 5 h. After addition of $N_2H_4 \cdot H_2O$ (24 µL, 0.49 mmol), stirring was continued for 16 h. The mixture was diluted with $CHCl_3$ (20 mL) and washed successively with 1 M aq. HCl (5.0 mL), 0.5 M aq. $CuSO_4$ (5.0 mL) and satd aq. $NaHCO_3$ (3 × 10 mL). The organic layer was dried over Na_2SO_4, filtererd and concentrated under reduced pressure to give an yellow oil. The crude product was reacted further in dry pyridine (1.0 mL) with acetic anhydride (0.5 mL) for 3 h at rt. The reaction mixture was diluted with $CHCl_3$ (10.0 mL) and washed with 0.5 M aq $CuSO_4$ (3 × 3 mL) and sat. aq. $NaHCO_3$ (3 × 5 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated. The crude product was purified by chromatography (petroleum ether/ethyl acetate 1:1) to yield **35** (14 mg, 34%) as a white foam. ^1H NMR (500 MHz, CD_3OD): δ 1.40 (s, 9H, $C(CH_3)_3$), 1.77 (t, J = 12.4 Hz, 1H, H-3a), 2.00, 2.13, 2.16 (3s, 9H, 3OAc), 2.67 (dd, J = 4.7, 12.6 Hz, 1H, H-3b), 2.86 (t, J = 2.4 Hz, 1H, C≡CH), 3.35 (dd, J = 6.0, 13.5 Hz, 1H, H-9a), 3.56 – 3.70 (m, 2H, H-5, H-9b), 3.83 (s, 3H, OMe), 4.03 (dd, J = 2.0, 10.7 Hz, 1H, H-6), 4.19, 4.35 (A, B of AB, J = 2.5, 15.8 Hz, 2H, H-1'), 4.79 (ddd, J = 4.7, 10.4, 12.0 Hz, 1H, H-4), 5.30 – 5.41 (m, 2H, H-7, H-8). ^{13}C NMR (CD_3OD, 125 MHz): δ 20.8, 20.9, 21.2 (3 OAc), 28.7 ($C(CH_3)_3$), 39.0 (C-3), 51.4 (C-5), 52.2 (C-9), 53.4 (OMe), 53.5 (C-1'), 69.5 (C-7), 70.8 (2C, C-4, C-8), 73.8 (C-6), 75.8 (C≡CH), 80.1 (C≡CH), 80.6 ($C(CH_3)_3$), 99.4 (C-2), 157.7 (CONH), 169.1, 171.5, 171.7, 171.8 (4 CO). ESI-MS calcd. for $C_{29}H_{33}Cl_2F_2NaN_2O_9$ $[M+Na]^+$: 593.21; Found 593.26.

Methyl [2-propynyl 4,7,8-tri-*O*-acetyl-5-amino-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulo-pyranosid] onate (36). Compound **35** (177 mg, 0.31 mmol) was dissolved in 4 M PhOH (5 mL, in dry DCM). After addition of 4 M TMSCl (5 mL, in dry DCM), the reaction mixture was stirred at rt for 2 h. After completion of the reaction, $CHCl_3$ was added and the organic layer was washed with satd aq. $NaHCO_3$ (3 × 10 mL) and water (1 × 10 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by chromatography (ethyl acetate/petroleum ether 1:2 then ethyl acetate/acetone 9:1) to yield **36** as a colourless oil (98 mg, 67%). ^1H NMR (500 MHz,

CD_2Cl_2): δ 1.63 (t, *J* = 12.2 Hz, 1H, H-3a), 1.97, 2.08, 2.10 (3s, 9H, 3OAc), 2.42 (t, *J* = 2.4 Hz, 1H, C≡C*H*), 2.49 (dd, *J* = 8.3, 10.9 Hz, 1H, H-5), 2.58 (dd, *J* = 4.5, 12.6 Hz, 1H, H-3b), 3.26 (dd, *J* = 5.0, 13.6 Hz, 1H, H-9a), 3.57 (dd, *J* = 2.8, 13.6 Hz, 1H, H-9b), 3.63 (dd, *J* = 1.0, 10.1 Hz, 1H, H-6), 4.04, 4.27 (dd, *J* = 2.5, 15.5 Hz, 1H, H-1'), 4.53 (ddd, *J* = 4.6, 10.0, 11.9 Hz, 1H, H-4), 5.29 (m, 1H, H-8), 5.42 (dd, *J* = 1.1, 8.5 Hz, 1H, H-7); ^{13}C NMR (CD_2Cl_2, 125 MHz): δ 21.1, 21.3, 21.4 (3 OAc), 37.2 (C-3), 52.3 (C-9), 52.9 (C-1'), 53.2 (C-5), 53.4 (OMe), 69.6 (2C, C-7, C-8), 71.8 (C-4), 72.1 (C≡*C*H), 74.8 (*C*≡CH), 75.9 (C-6), 117.2 (C-2), 170.4, 170.8, 170.9, 171.0 (4C, CO); ESI-MS calcd. for $C_{19}H_{26}N_4NaO_{10}$ $[M+Na]^+$: 493.15; found 493.21.

Methyl [2-propynyl -4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-5-fluoroacetamido -D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (37). Compound **36** (130 mg, 0.27 mmol) was dissolved in dry THF (3 mL) and cooled to 0 °C. Fluoroacetyl chloride (60 µL, 0.83 mmol) was added, followed by the addition of NEt_3 (190 µL, 1.35 mmol). The reaction mixture was allowed to come to rt and stirring was continued for 24 h. DCM (10 mL) was added and the organic layer was washed with satd aq. $NaHCO_3$ (3 × 2 mL) and water (1 × 2 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. The crude product was purified by chromatography (petroleum ether/ethyl acetate 1:1) to yield **37** (63 mg, 43%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.98 (t, *J* = 12.5 Hz, 1H, H-3a), 2.03, 2.15, 2.19 (3s, 9H, 3OAc), 2.46 (t, *J* = 2.1 Hz, 1H, C≡CH), 2.69 (dd, *J* = 4.5, 12.8 Hz, 1H, H-3b), 3.26 (dd, *J* = 5.4, 13.5 Hz, 1H, H-9a), 3.56 (dd, *J* = 2.6, 13.5 Hz, 1H, H-9b), 3.83 (s, 3H, OMe), 4.08 – 4.20 (m, 3H, H-5, H-6, H-1'a), 4.40 (B of AB, *J* = 2.3, 15.6 Hz, 1H, H-1'b), 4.57 – 4.86 (m, 2H, CH_2F), 4.93 (m, 1H, H-4), 5.25 – 5.40 (m, 2H, H-7, H-8), 6.19 (d, *J* = 8.6 Hz, 1H, NH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 21.0, 21.1, 21.2 (3 OAc), 38.1 (C-3), 48.7 (C-5), 51.1 (C-9), 53.3 (2C, OMe, C-1'), 67.9 (C-4), 68.7 (C-7), 69.4 (C-8), 72.7 (C-6), 74.8 (C≡*C*H), 79.0 (*C*≡CH), 79.5, 81.0 (d, *J* = 186 Hz, *C*H_2F), 98.3 (C-2), 168.3, 168.4, 170.3, 170.4, 170.9 (5 CO); ESI-MS calcd. for $C_{21}H_{27}FNaN_4O_{11}$ $[M+Na]^+$: 553.15; found m/z 553.12.

Methyl [2-propynyl 4,7,8-tri-*O*-acetyl-9-benzamido -3,5,9-trideoxy-5-fluoroacetamido-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (38a). Compound **37** (63 mg, 0.12 mmol) was dissolved in dry DCE (2 mL). Benzoyl chloride (55 µL, 0.47 mmol) and triphenylphosphine (70 mg, 0.26 mmol) were added successively and stirring was continued

for 24 h. DCM (5 mL) was added and the organic layer was washed with satd aq. $NaHCO_3$ (3 × 2 mL) and water (1 × 2 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. The crude product was purified by chromatography (0.5% gradient of MeOH in DCM) to yield **38a** (37 mg, 52%). ^{1}H NMR (500 MHz, CD_2Cl_2): δ 1.97 (m, 1H, H-3a) 2.08, 2.15, 2.21 (3s, 9H, 3OAc), 2.53 (t, *J* = 2.4 Hz, 1H, C≡C*H*), 2.75 (dd, *J* = 4.7, 12.7 Hz, 1H, H-3b), 3.08 (td, *J* = 4.3, 15.2 Hz, 1H, H-9a), 3.79 (s, 3H, OMe), 4.14 – 4.33 (m, 4H, H-5, H-6, H-9b, H-1'a), 4.43 (dd, *J* = 2.5, 15.6 Hz, 1H, H-1'b), 4.75 (m, 2H, CH_2F), 4.99 (ddd, *J* = 4.7, 10.4, 12.1 Hz, 1H, H-4), 5.24 (d, *J* = 9.5 Hz, 1H, H-7), 5.33 (m, 1H, H-8), 6.64 (d, *J* = 9.9 Hz, 1H, 5-NH), 7.04 (dd, *J* = 4.4, 7.4 Hz, 1H, 9-NH), 7.48 (m, 2H, CH_{ar}), 7.58 (m, 1H, CH_{ar}), 7.69 (m, 2H, CH_{ar}); ^{13}C NMR (CD_2Cl_2, 125 MHz): δ 21.0, 21.3, 21.4 (3 OAc), 38.2 (C-3), 38.5 (C-9), 48.9 (C-5), 53.1 (OMe), 53.3 (C-1'), 68.3 (C-7), 68.9 (C-8), 69.1 (C-4), 72.4 (C-6), 72.8 (C≡*C*H), 74.8 (*C*≡CH), 79.9 (CH_2F), 98.5 (C-2), 128.9, 129.0, 132.4, 134.8 (6C, C-Ar), 167.9, 168.4, 168.6, 168.9, 170.8, 172.3 (6 CO); ESI-MS calcd. for $C_{28}H_{33}FNaN_2O_{12}$ $[M+Na]^+$: 631.19; found m/z 631.18.

Methyl [2-propynyl 4,7,8-tri-*O*-acetyl-9-(4-chlorobenzamido) -3,5,9-trideoxy-5-fluoroacetamido-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (38b) Prepared from **37** (400 mg, 0.7531 mmol) and 4-chlorobenzoyl chloride (385 μL, 3.0124 mmol) according to procedure for compound 38a to afford **38b** (313mg, 65%) after chromatography. $[\alpha]_D^{20}$ -11.52 (*c* 1.41, DCM); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.96 (m, 1H, H-3a), 2.01, 2.11, 2.21 (3s, 9H, OAc), 2.42 (t, *J* = 2.4 Hz, 1H, C≡C*H*), 2.68 (dd, *J* = 4.6, 12.8 Hz, 1H, H-3b), 2.97 (dt, *J* = 4.0, 15.1 Hz, 1H, H-9a), 3.78 (s, 3H, OMe), 4.08 (m, 1H, H-6), 4.14 (dd, *J* = 2.4, 15.6 Hz, 1H, H-1'a), 4.17 – 4.30 (m, 2H, H-9b, H-5), 4.39 (dd, *J* = 2.5, 15.6 Hz, 1H, H-1'b), 4.58 – 4.79 (m, 2H, CH_2F), 4.86 (m, 1H, H-4), 5.13 (dd, *J* = 2.2, 9.8 Hz, 1H, H-7), 5.29 (m, 1H, H-8), 6.12 (dd, *J* = 3.2, 10.2 Hz, 1H, 5-NH), 6.89 (dd, *J* = 4.4, 8.0 Hz, 1H, 9-NH), 7.38 (d, *J* = 8.5 Hz, 2H, C_6H_4), 7.71 (d, *J* = 8.5 Hz, 2H, C_6H_4); ^{13}C NMR (126 MHz, $CDCl_3$) δ 20.98, 21.32, 21.34 (3 OAc), 38.11 (C-3), 38.86 (C-9), 48.89 (C-5), 53.13, 53.14 (C-1', OMe), 67.92 (C-7), 68.37 (C-8), 68.80 (C-4), 72.26 (C-6), 74.80 (C≡*C*H), 79.01 (*C*≡CH), 80.21 (d, $^2J_{C,F}$ = 186 Hz, CH_2F), 98.24 (C-2), 128.61, 129.04, 132.87, 137.95 (6C, C_6H_4), 166.58, 167.68, 170.63, 170.86, 172.47 (6C, C=O); ESI-MS *m/z* calcd for $C_{28}H_{32}ClFNaN_2NaO_{12}$ $[M+Na]^+$: 665.14; found: 665.15.

Lithium [2-propynyl 9-benzamido -3,5,9-trideoxy-5-fluoroacetamido -D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (39a). Compound **38a** (25 mg, 41 μmol) was dissolved in THF (4.5 mL) and LiOH (6.0 mg, 0.25 mmol in 0.5 mL water) was added. The reaction mixture was stirred at rt for 7 h. After neutralization with 7% aq. HCl the solvents were removed under reduced pressure and the crude product was purified by chromatography (1% gradient of water in DCM/MeOH 5:1) to yield **39a** (15 mg, 80%). ^{1}H NMR (500 MHz, CD_3OD): δ 1.59 (t, *J* = 11.6 Hz, 1H, H-3a), 2.70 (s, 1H, C≡CH), 2.80 (dd, *J* = 3.6, 11.9 Hz, 1H, H-3b), 3.42 (d, *J* = 8.7 Hz, 1H, H-7), 3.65 (m, 1H, H-9a), 3.68 – 3.85 (m, 4H, H-4, H-5, H-6, H-9b), 4.00 (m, 1H, H-8), 4.13, 4.40 (A, B of AB, *J* = 14.1 Hz, 2H, H-1'), 4.78 (m, 2H, CH_2F), 7.41 (t, *J* = 7.3 Hz, 2H, CH_{ar}), 7.48 (t, *J* = 7.1 Hz, 1H, CH_{ar}), 7.80 (d, *J* = 7.6 Hz, 2H, CH_{ar}); ^{13}C NMR (CD_3OD, 125 MHz): δ 42.2 (C-3), 44.3 (C-9), 53.2 (C-1'), 53.7 (C-5), 69.2 (C-4), 71.6 (C-8), 72.1 (C-7), 73.8 (C-6), 75.0 (C≡*C*H), 80.2, 81.7 (d, *J* = 181 Hz, CH_2F), 81.0 (*C*≡CH), 101.7 (C-2), 128.4, 129.6, 132.7, 135.6 (6C, C-Ar), 172.0, 178.5 (2 CO); ESI-MS calcd. for $C_{21}H_{24}FN_2O_9$ $[M-H]^-$: 467.15; found m/z 467.23.

Lithium [2-propynyl 9-(4-chlorobenzamido) -3,5,9-trideoxy-5-fluoroacetamido -D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (39b) Prepared from **38b** (313mg, 0.4868) according to procedure for compound 39a to afford **39b** (123 mg, 50%) as a white solid. $[\alpha]_D^{20}$ -17.43 (*c* 1.100, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.64 (t, *J* = 11.8 Hz, 1H, H-3a), 2.72 (t, *J* = 2.4 Hz, 1H, C≡CH), 2.85 (dd, *J* = 4.5, 12.3 Hz, 1H, H-3b), 3.45 (dd, *J* = 1.4, 8.9 Hz, 1H, H-7), 3.52 (m, 1H, H-9a), 3.73 – 3.92 (m, 4H, H-9b, H-4, H-5, H-6), 4.04 (td, *J* = 3.0, 8.4 Hz, 1H, H-8), 4.18 (dd, *J* = 2.3, 15.0 Hz, 1H, H-1'a), 4.44 (dd, *J* = 2.4, 15.0 Hz, 1H, H-1'b), 4.83 (d, *J* = 46.9 Hz, 2H, CH_2F), 7.46 (d, *J* = 8.5 Hz, 2H, C_6H_4), 7.82 (d, *J* = 8.5 Hz, 2H, C_6H_4), 8.35 (d, *J* = 5.1 Hz, 1H, NH); ^{13}C NMR (126 MHz, CD_3OD) δ 42.42 (C-3), 44.63 (C-9), 53.33 (C-1'), 53.73 (C-5), 69.45 (C-4), 71.56 (C-8), 72.33 (C-7), 73.95 (C-6), 75.09 (C≡*C*H), 81.09 (d, $^{2}J_{C,F}$ = 183.3 Hz, CH_2F), 81.14 (*C*≡CH), 129.82, 130.23, 134.56, 138.78 (6C, C_6H_4), 169.53, 172.18, 172.32 (C=O); ESI-MS *m/z* calcd for $C_{21}H_{24}ClFNaN_2NaO_9$ $[M+Na]^+$: 525.10; found: 525.04.

Sodium [3-(1-methyl-5-nitro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 5-acetamido -9-benzamido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (2). Prepared from **21**[17] (10 mg, 20 μmol) and **32** (6.5 mg, 30 μmol) according to general procedure C to afford **2** (12.3 mg, 80%) after chromatography as a yellow solid. $[\alpha]_D^{20}$ –19.8

(*c* 0.30, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ 1.62 (t, *J* = 11.5 Hz, 1H, H-3a), 2.0 (s, 3H, NHAc), 2.30 (m, 2H, H-2"), 2.80 (t, *J* = 7.0 Hz, 2H, H-3"), 2.88 (dd, *J* = 11.5, 3.0 Hz, 1H, H-3b), 3.43 (d, *J* = 9.0 Hz, 1H, H-7), 3.48 (dd, 1H, *J* = 13.5, 8.0 Hz, H-9a), 3.66 (d, *J* = 9.0 Hz, 1H, H-6), 3.82 (s, 3H, NCH_3), 3.81 - 3.71 (m, 3H, H-4, H-5, H-9b), 4.03 (dt, *J* = 8.5, 2.5 Hz, 1H, H-8), 4.43 (t, *J* = 7.0 Hz, 2H, H-1"), 4.67, 4.94 (d, *J* = 12.5 Hz, 2H, H-1'), 7.20 (s, 1H, CH_{ar}), 7.50 - 7.40 (m, 4H, CH_{ar}), 7.80 (d, *J* = 8.0 Hz, 2H, CH_{ar}), 7.96 (s, 1H, triazole-H), 8.06 (dd, *J* = 4.0, 1.5 Hz, 1H, CH_{ar}), 8.48 (d, *J* = 1.5 Hz, 1H, CH_{ar}); ^{13}C NMR (CD_3OD, 125 MHz): δ 22.5 (OAc), 22.6 (C-3"), 31.6 (C-2"), 33.2 (NCH_3), 42.6 (C-3), 44.6 (C-9), 50.8 (C-1"), 54.0 (C-5), 59.0 (C-1'), 69.6 (C-4), 71.3 (C-8), 72.5 (C-7), 74.4 (C-6), 102.0 (C-2), 110.6, 116.8, 117.3, 117.9, 125.4, 128.3, 131.6, 132.1, 135.8, l41.3, 142.2, 146.9 (16C, C-Ar), 170.3, 174.1, 175.4 (3 CO). HRMS calcd. for $C_{33}H_{38}N_7Na_2O_{11}$ $[M+Na]^+$: 754.2419; Found 754.2419.

Sodium [3-(1-ethyl-5-nitro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 5-acetamido -9-benzamido-3,5,9-trideoxy-D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (3). Prepared from **21** (10 mg, 20 μmol) and **33** (6.8 mg, 30 μmol) according to general procedure C to afford **3** (11 mg, 71%) after chromatography as a yellow solid. $[\alpha]_D^{20}$ –18.94 (*c* 0.27, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ 1.43 (t, *J* = 7.5 Hz, 3H, NCH_2CH_3), 1.62 (t, *J* = 11.5 Hz, 1H, H-3a), 2.0 (s, 3H, NHAc), 2.32 (m, 2H, H-2"), 2.81 (t, *J* = 7.5 Hz, 2H, H-3"), 2.88 (dd, *J* = 12.5, 4.0 Hz, 1H, H-3b), 3.43 (d, *J* = 9.0 Hz, 1H, H-7), 3.48 (dd, 1H, *J* = 13.5, 8.0 Hz, H-9a), 3.66 (m, 1H, H-6), 3.74 - 3.71 (m, 2H, H-5, H-4), 3.81 (dd, *J* = 13.5, 3.0 Hz, 1H, H-9b), 4.04 (td, *J* = 8.0, 3.0 Hz, 1H, H-8), 4.23 (q, *J* = 7.0 Hz, 2H, NCH_2), 4.44 (t, *J* = 7.0 Hz, 2H, H-1"), 4.67, 4.94 (d, *J* = 12.5 Hz, 2H, H-1'), 7.28 (s, 1H, CH_{ar}), 7.49 - 7.40 (m, 4H, CH_{ar}), 7.81 (d, *J* = 7.5 Hz, 2H, CH_{ar}), 7.96 (s, 1H, triazole-H), 8.06 (dd, *J* = 9.0, 2.0 Hz, 1H, CH_{ar}), 8.49 (d, *J* = 1.5 Hz, 1H, CH_{ar}); ^{13}C NMR (CD_3OD, 125 MHz): δ 15.8 (NCH_2CH_3), 22.6 (C-3"), 22.6 (NHAc), 31.6 (C-2"), 42.2 (NCH_2), 42.6 (C-3), 44.6 (C-9), 50.8 (C-1"), 54.1 (C-5), 59.0 (C-1'), 69.6 (C-4), 71.2 (C-8), 72.6 (C-7), 74.4 (C-6), 102.0 (C-2), 110.6, 116.9, 117.5, 117.8, 125.3, 128.3, 128.4, 129.5, 129.9, 132.5, 135.8, 140.4, 142.2, 146.9 (16C, C-Ar), 170.3, 174.1, 175.4 (3 CO); HRMS calcd. for $C_{34}H_{40}N_7Na_2O_{11}$ $[M+Na]^+$: 768.2576; Found 768.2580.

Sodium [3-(5-nitro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 5-acetamido -9-benzamido -3,5,9-trideoxy-D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (4) Prepared

from **21** (8.0 mg, 20 μmol) and **25a** (4.1 mg, 20 μmol) according to general procedure C to afford **4** (7.7 mg, 68%) after chromatography as a white solid. $[\alpha]_D^{20}$ –15.9 (*c* 0.2, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ 1.62 (t, *J* = 11.5 Hz, 1H, H-3a), 1.99 (s, 3H, NHAc), 2.28 (m, 2H, H-2"), 2.75 (t, *J* = 7.0 Hz, 2H, H-3"), 2.88 (dd, *J* = 12.5, 4.5 Hz, 1H, H-3b), 3.44 (d, *J* = 9.0 Hz, 1H, H-7), 3.51 (dd, 1H, *J* = 13.5, 8.0 Hz, H-9a), 3.67 (d, *J* = 9.0 Hz, 1H, H-6), 3.75-3.69 (m, 2H, H-4, H-5), 3.79 (dd, *J* = 13.5, 3.0 Hz, 1H, H-9b), 4.05 (dt, *J* = 8.0, 3.0 Hz, 1H, H-8), 4.38 (t, *J* = 7.0 Hz, 2H, H-1"), 4.68, 4.95 (d, *J* = 12.5 Hz, 2H, H-1'), 6.98 (t, *J* = 7.5 Hz, 1H, CH_{ar}), 7.08 - 7.04 (m, 2H, CH_{ar}), 7.32 (d, *J* = 8.0 Hz, 1H, CH_{ar}), 7.49 - 7.41 (m, 4H, CH_{ar}), 7.81 (d, *J* = 7.5 Hz, 2H, CH_{ar}), 7.94 (s, 1H, triazole-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 22.6 (NHAc), 22.9 (C-3"), 31.8 (C-2"), 42.6 (C-3), 44.6 (C-9), 50.9 (C-1"), 54.1 (C-5), 58.9 (C-1'), 69.7 (C-4), 71.2 (C-8), 72.6 (C-7), 74.4 (C-6), 102.0 (C-2), 112.2, 112.8, 114.6, 119.2, 119.5, 122.3, 123.3, 125.3, 128.3, 129.5, 132.5, 135.9, 146.7 (16C, C-Ar), 170.3, 174.1, 175.4 (3 CO); HRMS calcd. for $C_{32}H_{37}N_6Na_2O_9$ $[M+Na]^+$: 695.2412; Found 695.2413.

Sodium [3-(5-methoxy-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 5-acetamido -9-benzamido-3,5,9-trideoxy-D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (5). Prepared from **21** (10 mg, 20 μmol) and **25b** (5.8 mg, 30 μmol) according to general procedure C to afford **5** (8.2 mg, 70%) after chromatography as white solid. $[\alpha]_D^{20}$ –24.30 (*c* 0.23, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ 1.63 (t, *J* = 11.5 Hz, 1H, H-3a), 2.0 (s, 3H, NHAc), 2.27 (m, 2H, H-2"), 2.72 (t, *J* = 7.0 Hz, 2H, H-3"), 2.88 (dd, *J* = 13.5, 4.0 Hz, 1H, H-3b), 3.44 (d, *J* = 9.0 Hz, 1H, H-7), 3.52 (dd, 1H, *J* = 13.5, 8.0 Hz, H-9a), 3.66 (d, *J* = 9.0 Hz, 1H, H-6), 3.80 (s, 3H, OMe), 3.80 - 3.73 (m, 3H, H-4, H-5, H-9b), 4.05 (m, 1H, H-8), 4.39 (t, *J* = 7.0 Hz, 2H, H-1"), 4.68, 4.95 (d, *J* = 12.5 Hz, 2H, H-1'), 6.73 (d, *J* = 8.5 Hz, 1H, CH_{ar}), 6.95 (s, 1H, CH_{ar}), 7.02 (s, 1H, CH_{ar}), 7.20 (d, *J* = 8.5 Hz, 1H, CH_{ar}), 7.50 - 7.42 (m, 3H, CH_{ar}), 7.81 (d, *J* = 7.5 Hz, 2H, CH_{ar}), 7.95 (s, 1H, triazole-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 22.6 (NHAc), 22.9 (C-2"), 31.7 (C-3"), 42.6 (C-3), 44.5 (C-9), 50.9 (C-1"), 54.0 (C-5), 56.3 (OCH_3), 58.9 (C-1'), 69.7 (C-4), 71.2 (C-8), 72.5 (C-7), 74.4 (C-6), 101.1 (Ar-H), 102.0 (C-2), 111.4, 112.6, 112.9, 114.3, 124.1, 125.4, 128.3, 128.8, 129.5, 132.5, 133.4, 135.8, 146.7, 154.9 (16C, C-Ar), 170.3, 174.1, 175.4 (3 CO); HRMS calcd. for $C_{33}H_{39}N_6Na_2O_{10}$ $[M+Na]^+$: 725.2518; Found .725.2522.

Sodium [3-(5-trifluoromethyl-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 5-acetamido -9-benzamido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (6). Prepared from **21** (10 mg, 20 μmol) and **25o** (7 mg, 30 μmol) according to general procedure C to afford **6** (7.7 mg, 67%) after chromatography as white solid. $[\alpha]_D^{20}$ –21.38 (*c* 0.14, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ 1.62 (t, *J* = 11.5 Hz, 1H, H-3a), 2.0 (s, 3H, NHAc), 2.29 (m, 2H, H-2”), 2.79 (t, *J* = 7.0 Hz, 2H, H-3”), 2.87 (dd, *J* = 11.5, 3.5 Hz, 1H, H-3b), 3.44 (dd, *J* = 9.0, 2.0 Hz, 1H, H-7), 3.50 (dd, 1H, *J* = 13.5, 8.0 Hz, H-9a), 3.66 (m, 1H, H-6), 3.77 - 3.68 (m, 2H, H-4, H-5), 3.80 (dd, *J* = 13.5, 3.5 Hz, 1H, H-9b), 4.05 (td, *J* = 8.5, 3.0 Hz, 1H, H-8), 4.41 (t, *J* = 7.0 Hz, 2H, H-1’’), 4.69, 4.95 (d, *J* = 12.0 Hz, 2H, H-1’), 7.21 (s, 1H, CH_{ar}), 7.33 (m, 1H, CH_{ar}), 7.50-7.41 (m, 4H, CH_{ar}), 7.81 (m, 3H, CH_{ar}), 7.97 (s, 1H, triazole-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 22.6 (C-2”), 22.6 (NHAc) 31.6 (C-3”), 42.6 (C-3), 44.5 (C-9), 50.8 (C-1”), 54.1 (C-5), 58.9 (C-1’), 69.7 (C-4), 71.2 (C-8), 72.6 (C-7), 74.4 (C-6), 102.0 (C-2), 112.7, 115.8, 116.9 (q, *J* = 4.25 Hz), 118.8 (q, *J* = 3.5 Hz), 121.9 (q, *J* = 31.25 Hz), 125.4, 125.6, 127.9, 128.2, 129.5, 132.5, 135.8, 139.6, 146.8 (16C, C-Ar), 170.3, 174.2, 175.4 (3 CO); HRMS calcd for $C_{33}H_{36}F_3N_6Na_2O_9$ $[M+Na]^+$: 763.2286; Found 763.2295.

Sodium [3-(6-cyclopropylamide)-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 5-acetamido -9-benzamido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosid]onate (7). Prepared from **21** (6 mg, 10 μmol) and **25l** (4 mg, 20 μmol) according to general procedure C to afford **7** (6.1 mg, 65%) after chromatography as a white solid. $[\alpha]_D^{20}$ –15.3 (*c* 0.17, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ 1.15 - 1.07 (m, 4H, $CH(CH_2)_2$), 1.63 (t, *J* = 11.5 Hz, 1H, H-3a), 2.0 (s, 3H, NHAc), 2.30 (m, 2H, H-2”), 2.79 (t, *J* = 7.0 Hz, 2H, H-3”), 2.94 - 2.88 (m, 2H, $CH(CH_2)_2$, H-3b), 3.44 (dd, *J* = 9.0, 1.5 Hz, 1H, H-7), 3.51 (dd, 1H, *J* = 14.0, 8.0 Hz, H-9a), 3.68 (m, 1H, H-6), 3.78 - 3.72 (m, 2H, H-5, H-4), 3.80 (dd, *J* = 13.5, 3.5 Hz, 1H, H-9b), 4.05 (td, *J* = 8.0, 3.0 Hz, 1H, H-8), 4.42 (t, *J* = 7.0 Hz, 2H, H-1”), 4.69, 4.96 (d, *J* = 12.5 Hz, 2H, H-1’), 7.32 (s, 1H, CH_{ar}), 7.42 (t, *J* = 7.5 Hz, 1H, CH_{ar}), 7.50 (t, *J* = 7.5 Hz, 2H, CH_{ar}), 7.60 (d, *J* = 8.5 Hz, 1H, CH_{ar}), 7.75 (dd, *J* = 8.5, 1.5 Hz, 1H, CH_{ar}), 7.82 (m, 2H, CH_{ar}), 7.98 (s, 1H, CH_{ar}), 8.12 (s, 1H, triazole-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 11.9 ($CH(CH_2)_2$), 17.8 ($CH(CH_2)_2$), 22.6 (NHAc), 22.7 (C-3”), 31.8 (C-2”), 42.6 (C-3), 44.6 (C-9), 50.8 (C-1”), 54.1 (C-4), 59.0 (C-1’), 69.7 (C-5), 71.2 (C-8), 72.5 (C-7), 74.4 (C-6), 102.0 (C-2), 113.5 (16C, C-Ar), 115.3, 119.2, 119.7, 125.4, 128.1, 128.3, 129.5, 132.4, 132.5,

135.8, 137.6, 146.9 (C-Ar), 170.3, 174.2, 175.4, 203.5 (4 CO); HRMS calcd. for $C_{36}H_{41}N_6Na_2O_{10}$ $[M+Na]^+$: 763.2674, found 763.2673.

Sodium [3-(4-chlorobenzyl amide) -1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 5-acetamido -9-benzamido-3,5,9-trideoxy-D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (8). Prepared from **21** (6 mg, mmol) and **25m** (6.8 mg, mmol) according to general procedure C to afford **8** (4.8 mg, 47%) after chromatography as a pale yellow solid. $[\alpha]_D^{20}$ –11.99 (*c* 0.21, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ 1.62 (t, *J* = 11.5 Hz, 1H, H-3a), 2.0 (s, 3H, NHAc), 2.31 (m, 2H, H-2"), 2.80 (t, *J* = 7.0 Hz, 2H, H-3"), 2.89 (dd, *J* = 12.0, 4.0 Hz, 1H, H-3b), 3.42 (dd, *J* = 9.0, 2.0 Hz, 1H, H-7), 3.51 (dd, 1H, *J* = 13.5, 8.0 Hz, H-9a), 3.66 (m, 1H, H-6), 3.75 - 3.71 (m, 2H, H-4, H-5), 3.79 (dd, *J* = 13.5, 3.0 Hz, 1H, H-9b), 4.04 (td, *J* = 8.0, 3.0 Hz, 1H, H-8), 4.42 (t, *J* = 7.0 Hz, 2H, H-1"), 4.69, 4.96 (d, *J* = 12.5 Hz, 2H, H-1'), 7.35 (s, 1H, CH_{ar}), 7.41 (m, 2H, CH_{ar}), 7.51 - 7.48 (m, 2H, CH_{ar}), 7.56 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.64 (d, *J* = 8.5 Hz, 1H, CH_{ar}), 7.84 - 7.75 (m, 5H, CH_{ar}), 7.98 (s, 1H, triazole-H); ^{13}C NMR (CD_3OD, 125 MHz): δ 22.6 (NHAc), 22.7 (C-3"), 31.7 (C-2"), 42.6 (C-3), 44.6 (C-9), 50.8 (C-1"), 54.1 (C-5), 59.0 (C-1'), 69.7 (C-4), 71.2 (C-8), 72.6 (C-7), 74.4 (C-6), 102.1 (C-2), 115.5, 116.1, 119.3, 121.6, 125.4, 128.3, 129.5, 131.0, 135.6, 137.2, 138.7, 139.1, 147.0 (22C, C-Ar), 170.4, 174.1, 175.4, 198.2 (4 CO); HRMS calcd. for $C_{39}H_{40}ClN_6Na_2O_{10}$ $[M+Na]^+$: 833.2284; Found 833.2284.

Sodium [3-(5-nitro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-benzamido-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (9). Prepared from **39a** (15 mg, 30 μmol) and **25n** (12 mg, 45 μmol) according to general procedure C to afford **9** (3.7 mg, 16%) after chromatography as yellow solid. $[\alpha]_D^{20}$ –20.58 (*c* 1.00, MeOH); ^{1}H NMR (500 MHz, D_2O): δ 1.64 (t, *J* = 12.1 Hz, 1H, H-3a), 2.16 – 2.23 (m, 2H, H-2''), 2.61 (dd, *J* = 6.5, 11.9 Hz, 2H, H-3''), 2.69 (dd, *J* = 4.8, 12.5 Hz, 1H, H-3b), 3.34 (dd, *J* = 8.3, 14.0 Hz, 1H, H-9a), 3.52 (dd, *J* = 1.3, 8.6 Hz, 1H, H-7), 3.68 – 3.77 (m, 1H, H-4), 3.77 – 3.91 (m, 3H, H-6, H-8, H-9b), 3.95 (t, *J* = 10.1 Hz, 1H, H-5), 4.25 – 4.36 (m, 3H, H-1'a, H-1''), 4.55 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 4.92 (m, 1H, CH_2F), 7.04 (s, 1H, CH_{ar}), 7.23 (d, *J* = 9.0 Hz, 1H, CH_{ar}), 7.33 (t, *J* = 7.7 Hz, 2H, CH_{ar}), 7.43 (t, *J* = 7.4 Hz, 1H, CH_{ar}), 7.58 (s, 1H, CH_{ar}), 7.61 (d, *J* = 7.4 Hz, 2H, CH_{ar}), 7.78 (dd, *J* = 1.9, 8.9 Hz, 1H, CH_{ar}), 8.06 (d, *J* = 1.8 Hz, 1H, CH_{ar});^{13}C NMR (D_2O, 125 MHz): δ 28.9 (C-3''), 40.2 (C-2''), 42.5 (C-9), 50.0 (C-1''), 51.1 (C-5), 57.0 (C-1'), 67.8 (C-4), 69.8 (C-7), 70.0 (C-8), 72.0 (C-6),

78.9, 80.3 (d, *J* = 190.2, *C*H_2F), 111.0, 116.8, 125.0, 126.0, 126.7, 128.4, 131.9, 133.3 (15C, C-Ar), (3C, CO); HRMS calcd. for $C_{32}H_{35}FN_7Na_2O_{11}$ $[M+Na]^+$: 758.2174; found m/z 758.2176.

Sodium [3-(5-nitro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chloro-benzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (10). Prepared from **39b** (18.3 mg, 0.0364 mmol) and **25n** (13.3 mg, 0.0546 mmol) according to general procedure C to afford **10** (19 mg, 70%). $[\alpha]_D^{20}$ -21.558 (*c* 1.000, MeOH); $_1$H NMR (500 MHz, CD_3OD) δ 1.65 (t, *J* = 11.9 Hz, 1H, H-3a), 2.30 (p, *J* = 7.1 Hz, 2H, H-2"), 2.80 (t, J = 7.36 Hz, 2H, H-3"), 2.87 (dd, *J* = 3.9, 12.1 Hz, 1H, H-3b), 3.44 – 3.52 (m, 2H, H-9a, H-7), 3.78 – 3.95 (m, 4H, H-9b, H-4, H-5, H-6)), 4.03 (m, 1H, H-8), 4.43 (t, *J* = 6.8 Hz, 2H, H-1"), 4.68 (A of AB, *J* = 12.2 Hz, 1H, H-1'a), 4.80 (m, 1H, CH_2F), 4.94 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 7.27 (s, 1H, CH_{ar}), 7.42 (dd, *J* = 8.8, 11.0 Hz, 3H, CH_{ar}), 7.79 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.97 (s, 1H, CH_{ar}), 8.01 (dd, *J* = 2.1, 9.0 Hz, 1H, CH_{ar}), 8.48 (d, *J* = 2.1 Hz, 1H, CH_{ar}); 13C NMR (126 MHz, MeOD) δ 22.73 (C-3"), 31.71 (C-2"), 42.60 (C-3), 44.71 (C-9), 50.97 C-1"), 53.78 (C-5), 59.02 (C-1'), 69.57 (C-4), 71.69 (C-8), 72.44 (C-7), 73.99 (C-6), 81.12 (d, $^2J_{C,F}$ = 183.0 Hz, CH_2F), 112.57, 116.75, 117.73, 117.99, 127.29, 128.03, 129.77, 130.20, 134.51, 138.75, 141.37, 142.37 (15C, C-AR) 169.40, 172.17, 172.32 (C=O); ESI-MS *m/z* calcd for $C_{32}H_{35}ClFN_7NaO_{11}$ $[M+H]^+$: 770.20; found: 770.37.

Sodium [3-(5-chloro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (11). Prepared from **39b** (30 mg, 0.0591 mmol) and **25c** (20.8 mg, 0.0886 mmol) according to general procedure C to afford **11** (27 mg, 60%). $[\alpha]_D^{20}$ -21.024 (*c* 1.080, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.61 (m, 1H, H-3a), 2.23 (p, *J* = 7.1 Hz, 2H, H-2"), 2.69 (t, *J* = 7.3 Hz, 2H, H-3"), 2.88 (dd, *J* = 4.6, 12.2 Hz, 1H, H-3b), 3.39 – 3.59 (m, 2H, H-9a, H-7), 3.74 – 3.97 (m, 4H, H-9b, H-4, H-5, H-6), 4.03 (td, *J* = 3.0, 8.5 Hz, 1H, H-8), 4.37 (t, *J* = 7.0 Hz, 2H, H-1"), 4.68 (A of AB, *J* = 12.2 Hz, 1H, H-1'a), 4.84 (d, *J* = 46.7 Hz, 2H, CH_2F), 4.95 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 7.02 (dd, *J* = 2.0, 8.6 Hz, 1H, CH_{ar}), 7.09 (s, 1H, CH_{ar}), 7.28 (d, *J* = 8.6 Hz, 1H, CH_{ar}), 7.40 (d, *J* = 8.6 Hz, 2H, CH_{ar}), 7.44 (d, *J* = 1.9 Hz, 1H, CH_{ar}), 7.79 (d, *J* = 8.6 Hz, 2H, CH_{ar}), 7.91 (s, 1H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 22.86 (C-3"), 31.79 (C-2"), 42.64 (C-3), 44.64 (C-9), 50.98 (C-1"), 53.72 (C-5), 58.98 (C-1'), 69.59 (C-4), 71.76 (C-8), 72.38 (C-7), 73.96 (C-6), 81.12 (d, $^2J_{C,F}$ = 183.2 Hz,

CH_2F), 102.16, 113.63, 114.53, 118.79, 122.56, 125.28, 125.44, 125.47, 129.76, 130.19, 134.53, 136.70, 138.72, 146.78 (15C, C-Ar), 169.39, 172.14, 172.28 (C=O); ESI-MS *m/z* calcd for $C_{32}H_{35}Cl_2FN_6NaO_9$ $[M+H]^+$: 759.17; found: 759.28.

Sodium [3-(6-chloro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (12). Prepared from **39b** (16 mg, 0.0327 mmol) and **25f** (11.3 mg, 0.0482 mmol) according to general procedure C to afford **12** (19 mg, 79%). $[\alpha]_D^{20}$ -24.310 (*c* 1.067, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.65 (t, *J* = 12.0 Hz, 1H, H-3a), 2.28 (p, *J* = 7.1 Hz, 2H, H-2"), 2.88 (dd, *J* = 4.7, 12.3 Hz, 1H, H-3b), 2.95 (t, *J* = 7.4 Hz, 2H, H-3"), 3.40 – 3.57 (m, 2H, H-9a, H-7), 3.77 – 3.87 (m, 3H, H-9b, H-4, H-6), 3.92 (m, 1H, H-5), 4.04 (td, *J* = 3.1, 8.4 Hz, 1H, H-8), 4.41 (t, *J* = 6.9 Hz, 2H, H-1"), 4.67 (A of AB, *J* = 12.2 Hz, 1H, H-1'a), 4.84 (d, *J* = 47.0 Hz, 2H, CH_2F), 4.95 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 6.92 (d, *J* = 7.5 Hz, 1H, CH_{ar}), 6.98 (t, *J* = 7.8 Hz, 1H, CH_{ar}), 7.08 (s, 1H, CH_{ar}), 7.25 (d, *J* = 8.0 Hz, 1H, CH_{ar}), 7.41 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.79 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.95 (s, 1H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 24.32 (C-3"), 33.67 (C-2"), 42.71 (C-3), 44.66 (C-9), 50.95 (C-1"), 53.72 (C-5), 59.01 (C-1') , 69.66 (C-4), 71.70 (C-8), 72.45 (C-7), 73.95 (C-6) 81.12 (d, $^2J_{C,F}$ = 183.3 Hz, CH_2F), 102.17, 111.50, 115.07, 120.67, 122.99, 125.15, 125.46, 125.56, 126.86, 129.77, 130.18, 134.57, 138.72, 140.00, 146.86 (C-Ar), 169.41, 172.15, 172.30, (CO); ESI-MS *m/z* calcd for $C_{32}H_{35}Cl_2FN_6NaO_9$ $[M+H]^+$: 759.16; found: 759.22.

Sodium [3-(7-chloro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (13). Prepared from **39b** (12 mg, 0.0241 mmol) and **25i** (8.5 mg, 0.0302 mmol) according to general procedure C to afford **13** (15 mg, 83%). $[\alpha]_D^{20}$ -22.60 (*c* 1.00, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.65 (t, *J* = 11.9 Hz, 1H, H-3a), 2.28 (p, *J* = 7.2 Hz, 2H, H-2"), 2.74 (t, *J* = 7.4 Hz, 2H, H-3"), 2.88 (dd, *J* = 4.7, 12.3 Hz, 1H, H-3b), 3.43 – 3.53 (m, 2H, H-9a, H-7), 3.74 – 3.88 (m, 3H, H-9b, H-4, H-6), 3.92 (m, 1H, H-5), 4.03 (td, *J* = 3.1, 8.4 Hz, 1H, H-8), 4.40 (t, *J* = 7.0 Hz, 2H, H-1"), 4.69 (A of AB, *J* = 12.3 Hz, 1H, H-1'a), 4.84 (d, *J* = 47.0 Hz, 2H, CH_2F), 4.96 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 6.97 (t, *J* = 7.7 Hz, 1H, CH_{ar}), 7.09 (d, *J* = 7.5 Hz, 1H, CH_{ar}), 7.14 (s, 1H), 7.38 – 7.47 (m, 3H, CH_{ar}), 7.76 – 7.83 (m, 2H, CH_{ar}), 7.96 (s, 1H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 23.04 (C-3"), 31.85 (C-2"), 42.72 (C-3), 44.68 (C-9), 50.96 (C-1"), 53.73 (C-5), 59.05 (C-1'), 69.66 (C-4),

71.67 (C-8), 72.47 (C-7), 73.97 (C-6), 81.12 (d, $^2J_{C,F}$ = 183.1 Hz, CH_2F), 115.98, 117.84, 118.37, 120.61, 121.94, 124.69, 125.50, 129.78, 130.19, 130.55, 134.59, 135.23, 138.72 (15C, C-Ar), 169.39, 172.16, 172.31 (CO); ESI-MS *m/z* calcd for $C_{32}H_{35}Cl_2FN_6NaO_9$ $[M+H]^+$: 759.16; found: 759.30.

Sodium [3-(5-methyl-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (14). Prepared from **39b** (16 mg, 0.0327 mmol) and **25e** (10.6 mg, 0.0490 mmol) according to general procedure C to afford **14** (18 mg, 75%). $[\alpha]_D^{20}$ -23.215 (*c* 1.00, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.65 (t, *J* = 11.9 Hz, 1H, H-3a), 2.25 – 2.29 (m, 2H, H-2"), 2.46 (s, 3H, CH_3), 2.74 (t, *J* = 7.3 Hz, 2H, H-3"), 2.88 (dd, *J* = 4.7, 12.3 Hz, 1H, H-3b), 3.44 – 3.53 (m, 2H, H-9a, H-7), 3.76 – 3.87 (m, 3H, H-9b, H-4, H-6), 3.95 (m, 1H, H-5), 4.03 (td, *J* = 2.9, 8.5 Hz, 1H, H-8), 4.37 (t, *J* = 7.0 Hz, 2H, H-1"), 4.67 (A of AB, *J* = 12.2 Hz, 1H, H-1'a), 4.84 (d, *J* = 46.9 Hz, 2H, CH_2F), 4.95 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 6.83 – 6.94 (m, 2H, CH_{ar}), 7.05 (s, 1H, CH_{ar}), 7.31 (d, *J* = 7.5 Hz, 1H, CH_{ar}), 7.41 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.79 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.92 (s, 1H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 17.04 (CH_3), 23.18 (C-3"), 31.95 (C-2"), 42.71 (C-3), 44.65 (C-9), 51.03 (C-1"), 53.73 (C-5), 59.02 (C-1'), 69.64 (C-4), 71.72 (C-8), 72.44 (C-7), 73.95 (C-6), 81.12 (d, $^2J_{C,F}$ = 183.0 Hz, CH_2F), 114.99, 117.13, 120.00, 121.84, 122.97, 123.33, 125.47, 128.32, 129.77, 130.19, 134.58, 137.78, 138.71 (15C, C-Ar), 169.41, 172.15, 172.29, (CO); ESI-MS *m/z* calcd for $C_{33}H_{38}ClFN_6NaO_9$ $[M+H]^+$: 739.22; found: 739.26.

Sodium [3-(5-fluoro-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (15). Prepared from **39b** (13 mg, 0.0257 mmol) and **25d** (8.4 mg, 0.0385 mmol) according to general procedure C to afford **15** (10 mg, 50%). $[\alpha]_D^{20}$ -17.624 (*c* 0.680, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.65 (t, *J* = 12.0 Hz, 1H, H-3a), 2.26 (p, *J* = 7.2 Hz, 2H, H-2")), 2.71 (t, *J* = 7.3 Hz, 2H, H-3"), 2.88 (dd, *J* = 4.8, 12.3 Hz, 1H, H-3b), 3.42 – 3.54 (m, 2H, H-9a, H-7), 3.75 – 3.87 (m, 3H, H-9b, H-4, H-6), 3.91 (m, 1H, H-5), 4.04 (td, *J* = 3.1, 8.1 Hz, 1H, H-8), 4.39 (t, *J* = 7.0 Hz, 2H, H-1"), 4.68 (A of AB, *J* = 12.2 Hz, 1H, H-1'a), 4.84 (d, *J* = 47.0 Hz, 2H, CH_2F), 4.95 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 6.83 (td, *J* = 2.5, 9.1 Hz, 1H, CH_{ar}), 7.11 (s, 1H, CH_{ar}), 7.14 (dd, *J* = 2.5, 9.9 Hz, 1H, CH_{ar}), 7.27 (dd, *J* = 4.4, 8.8 Hz, 1H, CH_{ar}), 7.39 – 7.46 (m, 2H, CH_{ar}), 7.76 – 7.84 (m, 2H, CH_{ar}), 7.94 (s, 1H,

CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 23.03 (C-3"), 31.80 (C-2"), 42.77 (C-3), 44.72 (C-9), 51.03 (C-1"), 53.77 (C-5), 59.08 (C-1'), 69.71 (C-4), 71.71 (C-8), 72.51 (C-7), 74.01 (C-4), 81.12 (d, $^2J_{C,F}$ = 183.3 Hz, CH_2F), 102.22 (C-Ar), 104.02 (d, $^2J_{C,F}$ = 23.40 Hz, C-4_{indole}), 110.53 (d, $^2J_{C,F}$ = 26.48, C-6_{indole}), 113.17 (d, $^3J_{C,F}$ = 9.71 Hz, C-7_{indole}), 114.91 (d, $^4J_{C,F}$ = 4.78 Hz, C-8_{indole}), 125.53, 125.63 (C-Ar), 129.00 (d, $^3J_{C,F}$ = 9.57 Hz, C-3_{indole}), 129.83, 130.24, 134.65, 134.97, 138.76, 146.93 (C-Ar), 158.99 (d, $^1J_{C,F}$ = 232.10 Hz, C-5_{indole}), 169.45, 172.20, 172.34, (CO); ESI-MS *m/z* calcd for $C_{32}H_{35}ClF_2N_6NaO_9$ $[M+H]^+$: 743.19; found: 743.34.

Sodium [3-(5-methoxy-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (16). Prepared from **39b** (10 mg, 0.0200 mmol) and **25b** (6.9 mg, 0.0300 mmol) according to general procedure C to afford **16** (8.6 mg, 61%). $[\alpha]_D^{20}$ -20.529 (*c* 0.667, MeOH); ^1H NMR (500 MHz, CD_3OD) δ 1.64 (t, *J* = 11.9 Hz, 1H, H-3a), 2.27 (p, *J* = 7.1 Hz, 2H, H-2"), 2.72 (t, *J* = 7.3 Hz, 2H, H-3"), 2.88 (dd, *J* = 4.7, 12.2 Hz, 1H, H-3b), 3.43 – 3.53 (m, 2H, H-9a, H-7), 3.81 (s, 3H, OCH_3), 3.77 – 3.87 (m, 3H, H-9b, H-4, H-6), 3.91 (m, 1H, H-5), 4.04 (td, *J* = 3.1, 8.4 Hz, 1H, H-8), 4.40 (t, *J* = 6.9 Hz, 2H, H-1"), 4.68 (A of AB, *J* = 12.2 Hz, 1H, H-1'a), 4.84 (d, *J* = 47.0 Hz, 2H, CH_2F), 4.96 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 6.73 (dd, *J* = 2.4, 8.8 Hz, 1H, CH_{ar}), 6.95 (d, *J* = 2.3 Hz, 1H, CH_{ar}), 7.02 (s, 1H, CH_{ar}), 7.21 (d, J = 8.8 Hz, 1H, CH_{ar}), 7.42 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.80 (t, *J* = 7.1 Hz, 2H, CH_{ar}), 7.94 (s, 1H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 23.03 (C-3"), 31.85 (C-2"), 42.74 (C-3), 44.70 (C-9), 51.03 (C-1"), 53.73 (C-5), 56.51 (OCH_3), 59.05 (C-1'), 69.68 (C-4), 71.65 (C-8), 72.50 (C-7), 73.97 (C-6), 81.11 (d, $^2J_{C,F}$ = 183.3 Hz, CH_2F), 101.34, 112.76, 113.06, 114.44, 124.27, 125.51, 128.98, 129.79, 130.20, 133.62, 134.61, 138.71, 155.07 (15C, C-Ar), 169.40, 172.16, 172.30 (CO); ESI-MS *m/z* calcd for $C_{33}H_{38}ClFN_6NaO_{10}$ $[M+H]^+$: 755.12; found: 755.27.

Sodium [3-(5-isopropyl-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (17). Prepared from **39b** (17.5 mg, 0.0349 mmol) and **25g** (12.7 mg, 0.0524 mmol) according to general procedure C to afford **17** (13 mg, 50%). $[\alpha]_D^{20}$ -18.50 (*c* 0.967, MeOH); ^1H NMR (500 MHz, CH_3OD) δ 1.28 (d, *J* = 6.9 Hz, 6H, CH_3), 1.65 (t, *J* = 11.8 Hz, 1H, H-3a), 2.28 (p, *J* = 7.1 Hz, 2H, H-2"), 2.74 (t, *J* = 7.3 Hz, 2H, H-3"), 2.89 (dd, *J* = 3.3, 11.8 Hz, 1H, H-3b), 2.95 (m, 1H, CH), 3.43 – 3.52 (m, 2H, H-9a, H-7), 3.77 – 3.87 (m,

3H, H-4, H-6, H-9b), 3.91 (m, 1H, H-5), 4.04 (td, *J* = 2.3, 8.3 Hz, 1H, H-8), 4.39 (t, *J* = 6.9 Hz, 2H, H-1"), 4.68 (A of AB, *J* = 11.9 Hz, 1H, H-1'a), 4.84 (d, *J* = 47.0 Hz, 2H, CH_2F), 4.95 (B of AB, *J* = 11.9 Hz, 1H, H-1'b), 6.98 (dd, *J* = 1.3, 8.4 Hz, 1H, CH_{ar}), 7.01 (s, 1H, CH_{ar}), 7.24 (d, *J* = 8.4 Hz, 1H, CH_{ar}), 7.29 (s, 1H, CH_{ar}), 7.41 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.79 (d, *J* = 8.5 Hz, 2H, C_6H_4), 7.95 (s, 1H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 23.08 (C-3"), 25.41 (2C, CH_3), 31.92 (C-2"), 35.74 (CH), 42.73 (C-3), 44.70 (C-9), 51.07 (C-1"), 53.73 (C-5), 59.05 (C-1'), 69.67 (C-4), 71.66 (C-8), 72.49 (C-7), 73.96 (C-6), 81.12 (d, $^2J_{C,F}$ = 183.2 Hz, CH_2F), 112.22, 114.39, 116.21, 121.58, 123.61, 128.75, 129.78, 130.20, 134.60, 137.03, 138.71, 140.44 (16C, C-Ar), 169.39, 172.15, 172.30 (CO); ESI-MS *m/z* calcd for $C_{35}H_{42}ClFN_6NaO_9$ $[M+H]^+$: 767.25; found: 767.33.

Sodium [3-(5-cyano-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (18). Prepared from **39b** (15.3 mg, 0.0305 mmol) and **25j** (10.3 mg, 0.0457 mmol) according to general procedure C to afford **18** (16 mg, 72%). $[\alpha]_D^{20}$ -22.12 (*c* 1.07, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.65 (t, *J* = 11.9 Hz, 1H, H-3a), 2.28 (p, *J* = 7.1 Hz, 2H, H-2"), 2.73 – 2.83 (m, 2H, H-3"), 2.88 (dd, *J* = 4.8, 12.3 Hz, 1H, H-3b), 3.48 (dd, *J* = 8.1, 13.9 Hz, 2H, H-9a, H-7), 3.76 – 3.88 (m, 3H, H-4, H-6, H-9b), 3.91 (m, 1H, H-5), 4.03 (td, *J* = 3.1, 8.3 Hz, 1H, H-8), 4.41 (t, *J* = 6.9 Hz, 2H, H-1"), 4.68 (A of AB, *J* = 12.3 Hz, 1H, H-1'a), 4.84 (d, *J* = 47.0 Hz, 2H, CH_2F), 4.95 (B of AB, *J* = 12.3 Hz, 1H, H-1'b), 7.23 (s, 1H, CH_{ar}), 7.35 (dd, *J* = 1.4, 8.4 Hz, 1H, CH_{ar}), 7.42 (d, *J* = 8.6 Hz, 2H, CH_{ar}), 7.46 (d, *J* = 8.4 Hz, 1H, CH_{ar}), 7.79 (d, *J* = 8.6 Hz, 2H, CH_{ar}), 7.95 (s, 2H, CH_{ar}); ^{13}C NMR (126 MHz, MeOD) δ 22.72 (C-3"), 31.73 (C-2"), 42.71 (C-3), 44.67 (C-9), 50.94 (C-1"), 53.73 (C-5), 59.03 (C-1'), 69.65 (C-4), 71.69 (C-8), 72.45 (C-7), 73.96 (C-6), 81.12 (d, $^2J_{C,F}$ = 183.2 Hz, CH_2F), 102.18, 102.27 (C-2, C-Ar), 113.62, 116.08, 122.17, 125.27, 125.40, 125.52, 126.31, 128.62, 129.78, 130.19, 134.57, 138.72, 140.12, 146.92 (CN, C-Ar), 169.38, 172.17, 172.31, (CO); ESI-MS *m/z* calcd for $C_{33}H_{35}ClFN_7NaO_9$ $[M+H]^+$: 750.20; found: 750.25.

Sodium [3-(5-methylsulfonyl -1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (19). Prepared from **39b** (9.6 mg, 0.0192 mmol) and **25k** (8 mg, 0.0287 mmol) according to general procedure C to afford **19** (15 mg, 66%). $[\alpha]_D^{20}$ -20.41 (*c* 0.60, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.64 (t, *J* = 12.0 Hz, 1H, H-3a), 2.32 (p, *J* =

7.1 Hz, 2H, H-2"), 2.82 (t, *J* = 7.4 Hz, 2H, H-3"), 2.88 (dd, *J* = 4.8, 12.3 Hz, 1H, H-3b), 3.12 (s, 3H, CH_3), 3.43 – 3.50 (m, 2H, H-9a, H-7), 3.77 – 3.86 (m, 3H, H-4, H-6, H-9b), 3.90 (m, 1H, H-5), 4.04 (td, *J* = 3.1, 8.3 Hz, 1H, H-8), 4.42 (t, *J* = 6.9 Hz, 2H, H-1"), 4.67 (A of AB, *J* = 12.3 Hz, 1H, H-1'a), 4.84 (d, *J* = 46.9 Hz, 2H, CH_2F), 4.94 (B of AB, *J* = 12.3 Hz, 1H, H-1'b), 7.28 (s, 1H, CH_{ar}), 7.42 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.53 (d, *J* = 8.6 Hz, 1H, CH_{ar}), 7.64 (dd, *J* = 1.7, 8.6 Hz, 1H, CH_{ar}), 7.80 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.94 (s, 1H, CH_{ar}), 8.14 (d, *J* = 1.4 Hz, 1H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 22.78 (C-3"), 31.70 (C-2"), 42.74 (C-3), 44.71 (C-9), 45.48 (CH_3), 50.94 (C-1"), 53.74 (C-5), 59.06 (C-1'), 69.68 (C-4), 71.65 (C-8), 72.50 (C-7), 73.97 (C-6), 81.12 (d, $^2J_{C,F}$ = 183.3 Hz, CH_2F), 102.18, 113.22, 116.75, 120.35, 120.86, 125.51, 126.67, 128.32, 129.79, 130.20, 131.82, 134.61, 138.71, 140.68, 146.96 (C-Ar), 169.38, 172.16, 172.31, (CO); ESI-MS *m/z* calcd for $C_{33}H_{38}ClFN_6NaO_{11}S$ $[M+H]^+$: 803.18; found: 803.25.

Sodium [3-(5,6-cyclopropyl-1*H*-indol-3-yl)propyl-[1,2,3]triazole-4-yl-methyl 9-(4-chlorobenzamido)-3,5,9-trideoxy-5-fluoroacetamido -D-*glycer*o-α-D-*galacto*-2-nonulopyranosid]onate (20). Prepared from **39b** (10 mg, 0.0191 mmol) and **25h** (6.9 mg, 0.0287 mmol) according to general procedure C to afford **20** (9 mg, 64%). $[\alpha]_D^{20}$ -24.540 (*c* 0.573, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 1.65 (t, *J* = 11.9 Hz, 1H, H-3a), 2.07 (p, *J* = 7.2 Hz, 2H, CH_2^{cyc}), 2.26 (p, *J* = 6.9 Hz, 2H, H-2"), 2.71 (t, *J* = 7.2 Hz, 2H, H-3"), 2.89 (dd, *J* = 4.7, 12.3 Hz, 1H, H-3b), 2.93 (t, *J* = 7.2 Hz, 4H, CH_2^{cyc}), 3.43 – 3.53 (m, 2H, H-9a, H-7), 3.76 - 3.86 (m, 3H, H-9b, H-4, H-6), 3.91 – (m, 1H, H-5), 4.03 (td, *J* = 3.0, 8.4 Hz, 1H, H-8), 4.38 (t, *J* = 7.0 Hz, 2H, H-1"), 4.68 (A of AB, *J* = 12.2 Hz, 1H, H-1'a), 4.84 (d, *J* = 47.0 Hz, 2H, CH_2F), 4.96 (B of AB, *J* = 12.2 Hz, 1H, H-1'b), 6.94 (s, 1H, CH_{ar}), 7.14 (s, 1H , CH_{ar}), 7.26 (s, 1H, CH_{ar}), 7.41 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.79 (d, *J* = 8.5 Hz, 2H, CH_{ar}), 7.93 (s, 1H, CH_{ar}); ^{13}C NMR (126 MHz, CD_3OD) δ 23.21 (C-3"), 27.89 (C^{cyc}), 31.92 (C-2"), 33.57, 33.89 (C^{cyc}), 42.73 (C-3), 44.70 (C-9), 51.06 (C-1"), 53.73 (C-5), 59.04 (C-1'), 69.67 (C-4), 71.66 (C-8), 72.50 (C-7), 73.97 (C-6), 81.11 (d, $^2J_{C,F}$ = 183.2 Hz, CH_2F), 107.62, 114.09, 114.11, 122.86, 125.53, 127.98, 129.78, 130.19, 134.59, 136.19, 138.12, 138.71, 139.48 (15C, C-Ar), 169.40, 172.16, 172.31 (CO); ESI-MS *m/z* calcd for $C_{35}H_{40}ClFN_6NaO_9$ $[M+H]^+$: 765.23; found: 765.25. 9494

Surface Plasmon Resonance. The SPR measurements were performed on a Biacore 3000 surface plasmon resonance based optical biosensor (Biacore AB, Sweden). Sensor chips (CM5), immobilization kits, maintenance supply and HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) were purchased from Biacore AB (HBS-EP ready-to-use; degassed and filtered). CM5 chips were preconditioned prior to usage by injecting a series of conditioning solutions. A flow rate of 50 µL/min was used and 2 × 20 µL of 50 mM NaOH, 10 mM HCl, 0.1% SDS and 100 mM H_3PO_4 were injected. The carboxy groups on the CM5 chip were activated for 10 min with a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.1 M 3-(*N*,*N*-dimethylamino)propyl-*N*-ethylcarbodiimide (EDC) at a flow rate of 10 µL/min. Polyclonal goat anti-human IgG (Fc specific) was purchased from Sigma (I2136, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). A sample and a reference surface were prepared sequentially or in parallel. For immobilizing, a 30 µg/mL solution of the polyclonal antibody diluted in acetate buffer (10 mM sodium acetate, pH 5.0) was then injected over the activated surface for 10 min at a flow rate of 10 µL/min. Densities around 13000 to 14000 RU were achieved. Flow cells were blocked with a 10 min injection of 1 M ethanolamine, pH 8.0. For capturing, $MAG_{d1\text{-}3}$-Fc solution (expressed and purified as decribed[27]) was diluted to a 30-40 µg/mL concentration using HBS-EP. Afterwards, $MAG_{d1\text{-}3}$-Fc was injected at a flow rate of 1 µL/min for 10 min. The surface was equilibrated over night at a flow rate of 5 µL/min, achieving densities around 3500 to 4000 RU. Tenfold dilution series were freshly prepared in eluent buffer immediately before use. All binding experiments were conducted at 25 °C at a flow rate of 20 µL/min. The samples were injected over 1 min followed by 1 min dissociation. Each sample was measured with a duplicate of one concentration, using a randomized concentration order. Several buffer samples were injected before the first concentration, and one blank between each concentration, which were used for the double blank referencing during data processing. Double referencing was applied to correct for bulk effects and other systematic artifacts. Data processing and equilibrium binding constant determinations were accomplished with Scrubber (BioLogic Software, Version 1.1g or 2.0c). Kinetic data were simultaneously fit using the non-linear regression program Clamp or Scrubber 2.0c.

CMC determination. 10 mM stock solutions in DMSO of the samples were prepared. Then, a dilution series in DMSO was prepared by a Hamilton Microlab Star robot (Hamilton AG, Bonaduz, Switzerland) and 5 µL of each well were transferred into buffer (0.05 M MOPSO, pH 6.5) to yield 50 µL of the 12 desired concentrations (between 0 and 1 mM). Before

starting the experiment, the Delta-8 instrument (Kibron Inc., Espoo, Finland) was calibrated with water. Finally, the 12 dilutions were measured and the results were analyzed with the Vector software (Kibron, version 4.02). The assay used for CMC determination was performed at F. Hoffmann-La Roche Ldt., Basel.

Isothermal Titration Calorimetry. ITC experiments were performed using a VP-ITC instrument from MicroCal, Inc. (Northampton, MA). The measurements were performed at 25 °C. Injections of 10 µL ligand solutions were added from a computer controlled 300 µL microsyringe at an interval of 5 min into the sample cell solution of MAG (cell volume 1.4512 mL) with stirring at 307 rpm. A control experiment was performed, where the identical ligand solutions were injected into buffer without protein. The enthalpogram showed negligible heat development, resulting from dilution effects. The assay buffer was HBS-E (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4). The concentration of MAG was 6.3 µM (determined by HPLC) and 100 µM antagonist was injected. The experimental data were fitted to a theoretical titration curve (one site binding model) using *Origin version 7* software (MicroCal), with ΔH (enthalpy change in kcal/mol), K_a (association constant in M^{-1}), and N (number of binding sites) as adjustable parameters. The quantity $c = K_a \cdot Mt(0)$, where Mt(0) is the initial macromolecule concentration, is of importance in titration microcalorimetry.[28] The experiment was performed with a *c* value of 48. Thermodynamic parameters were calculated from equation (1),

$$\Delta G = \Delta H - T\Delta S = RT\ln K_A = -RT\ln K_D \qquad (1)$$

where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively, T is the absolute temperature, and R = 1.98 cal/mol/K. For reasons of consistency the values were converted to kJ (1 cal = 4.1868 J).

Protein concentration determination by HPLC. The concentration of MAG was determined via HPLC against a standard curve of BSA at 210 nm using a Beckmann Gold system, with UV detection (180 nm). The column used was Poros R1/10 10 µm (100 × 2 mm, Dr. Maisch HPLC Markensäulen, po10.r1.s1002, Morvay Analytik GmbH). The running buffers were A: H_2O + 0.1% TFA and B: 90% MeCN + 0.09% TFA. All measurements were performed at 75 °C, applying a gradient of 20% to 90% running buffer B within 20 min at a flow rate of 0.2 mL/min.[32]

Molecular Modeling. Molecular-dynamic simulations (MD) using *Desmond*,[24] were performed to check the stability of the proposed binding modes along with the kinetic aspects of the binding. The complexes were simulated during 4.0 ns at 300 K using explicit solvent (TIP3P water) and sampled at 1.2 ps intervals. Those frames were then analyzed for hydrogen-bond contribution, π stacking and the **1** $-NO_2\cdots$Lys 67 interactions to complex stabilization.

FEP was employed to calculate the relative binding free energy (ΔΔG) between **1** and a corresponding mutant lacking the 5'-nitro group. The FEP was simulated for a total of 1.0 ns and divided in 12 λ windows using the SPC water model. In order to obtain more reliable affinity estimations, contribution of solvation to ΔΔG is explicitly included for both states. The ΔΔG is calculated as follows from the solvated states:

$$\Delta\Delta G = \Delta G[\text{complex}_{\mathbf{17b}}–\text{complex}_{\text{mutant}}] – \Delta G[\text{ligand}_{\mathbf{17b}}–\text{ligand}_{\text{mutant}}]$$
$$= (–2.32 \pm 0.10 \text{ kcal/mol}) – (–5.97 \pm 0.16 \text{ kcal/mol})$$
$$= +3.65 \pm 0.19 \text{ kcal/mol}$$

Acknowledgement.

We are greatly indebted to Frank Senner and Dr. Manfred Kansy, Roche, for allowing us to use their instruments for CMC determination.

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From a Library of MAG Antagonists to Nanomolar CD22 Ligands

Antagonists for CD22 were optimized and investigated with respect to their affinity and pharmacokinetic properties.

This part was published in *ChemMedChem*:
Stefanie Mesch, Katrin Lemme, Matthias Wittwer, Hendrik Koliwer-Brandl, Oliver Schwardt, Sørge Kelm and Beat Ernst, in the press.

Work performed by Stefanie Mesch:
Synthesis of CD22-Antagonsits and performance and evaluation of the Biacore assay.

DOI: 10.1002/cmdc.201100407

VIP

From a Library of MAG Antagonists to Nanomolar CD22 Ligands

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Siglec-2, also known as CD22, is involved in the regulation and survival of B-cells and has been successfully targeted in cell depletion therapies with antibody-based approaches. Sialic acid derivatives, already known to bind with high affinity to myelin-associated glycoprotein (MAG, Siglec-4), were screened for their binding affinity for CD22 by surface plasmon resonance. The best compound identified was further modified with various hydrophobic substituents at the 2-, 5-, and 9-positions of the sialic acid scaffold, leading to nanomolar derivatives, of which ligand **17b** shows the most promising pharmacodynamic and pharmacokinetic profiles. Isothermal titration calorimetry measurements demonstrate that the binding is enthalpy driven. Interestingly, the thermodynamic fingerprints reveal an excellent correlation between gains in enthalpy and compensation by increased entropy costs. Moreover, **17b** exhibits a residence time in the range of a few seconds, clearly prolonged relative to residence times typically observed for carbohydrate–lectin interactions. Finally, initial tests regarding drug-like properties of **17b** demonstrate the required high plasma protein binding yet a lack of oral availability, although its distribution coefficient (log *D*) is in the required range.

Introduction

Patients diagnosed with B-cell lymphomas can be effectively treated with the anti-CD20 antibody rituximab.[1] However, this therapy is not a cure, and especially for patients with indolent lymphoid malignancies novel treatments with alternative mechanisms of B-cell killing are required.[2] Numerous antibodies for B-cell depletion therapy are therefore under clinical development.[3] Two of them, the immunotoxins BL22[4] and CMC-544,[5] target CD22, a B-lymphocyte-specific receptor. CD22 (Siglec-2) is a member of the sialic acid binding immunoglobulin-like lectin (Siglec) family.[6] It is an inhibitory co-receptor for the B-cell receptor (BCR) and plays a crucial role in the regulation of activity,[7] homeostasis,[8] and survival of B-cells.[9] Upon BCR–antigen binding, tyrosine phosphorylation is induced,[10] which triggers further phosphorylation processes and finally leads to a dampening of the BCR-induced signal.[6,11] Apart from a few exceptions,[12] sialic acid binding sites of CD22 were shown to be effectively occupied by *cis* ligands, that is, ligands located on the same cell surface.[13] This interaction is important for the regulation of CD22 activity. Furthermore, it was shown that CD22 can also interact with ligands in *trans*,[14] that is, ligands located on other cells, to enable cell–cell communication. As an alternative to antibodies, sialosides that were shown to directly influence CD22 activity[15] initiated an intense search for high-affinity ligands.

The physiological ligands of Siglecs are gangliosides and sialidated glycoproteins.[16,17] CD22 recognizes sialic acid α2,6-linked to D-Gal, D-Gal*N*Ac (→**1**), or D-Glc*N*Ac. A decrease in the structural complexity yielded sialic acid derivatives **2**–**4**[15,18,19] (Figure 1), indicating that a biphenyl carboxamide moiety at the 9-position can improve affinity substantially. When this extension in the 9-position was applied to the disaccharide epitope, ligands such as **5** and **6** exhibiting sub-micromolar affinities were identified.[20] Finally, benzyl substituents at the reducing end of the disaccharide epitope led to a further improvement in affinity.[19,21]

When testing in B-cell based assays, a further aspect, the so-called density-dependent binding, must be considered.[22] In tests of monomeric sialosides (e.g., **7**[23] or **8**,[24] Figure 1) coupled to various supports such as a polyacrylamide backbone,[23] a glutamate cluster,[18] or a polymer obtained by ring-opening metathesis,[25] a substantial increase in binding affinity relative to monovalent sialosides was observed.[18,26] Interestingly, with a multivalent presentation, *cis* interactions could be abolished without precedent treatment with sialidase to remove masking by *cis* ligands.[13] Additionally, O'Reilly et al. showed that bifunctional ligands comprising a ligand of CD22 linked to an antigen can self-assemble by antibody triggering on B-cell surfaces and are able to overcome *cis* interactions as well.[24] Based on the current state of knowledge, an oligovalent presentation of glycan ligands is required for a successful competition with *cis* ligands.[27] However, further investigation is required to clarify whether an oligomeric display is mandatory to compete with *cis* ligands, or if monomeric high-affinity ligands could success-

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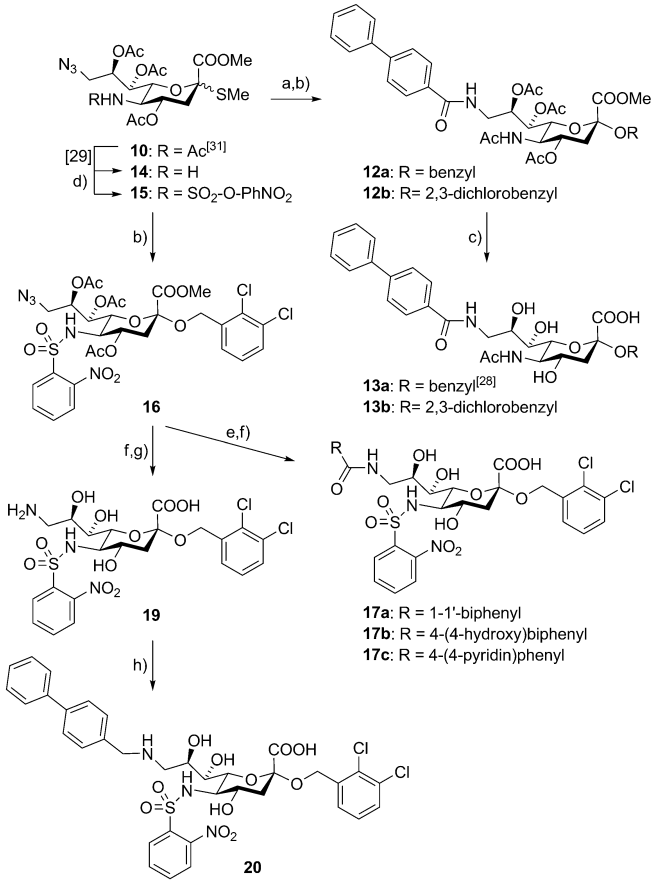
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201100407.



Scheme 1. *Reagents and conditions:* a) 4-phenylbenzoyl chloride, PPh_3, DCE (77%); b) ROH, NIS, TfOH, CH_3CN [**12a**: 70%, **12b**: 50%, **16**: 76%]; c) 10% aq NaOH [**13a**: 51%, **13b**: 25%]; d) *o*-nosyl-Cl, NEt_3, DMAP, CH_2Cl_2 (78%); e) RCOCl, PPh_3, DCE; f) LiOH, H_2O, THF [**17a–c**: 6–19% for two steps, →**18**: 52%]; g) PPh_3, H_2O, NEt_3, 50 °C (13%); h) 1-(bromomethyl)-4-phenylbenzene, K_2CO_3, THF (12%).

of the ligand–CD22 interaction, but also the pharmacokinetic properties of the best representatives.

Starting from sialyl donor **10**,[31] test compound **13b** was obtained according to published procedures.[28,29] The *o*-nosyl derivatives were synthesized according to a different approach (Scheme 1). After cleavage of the *N*-acetate (→**14**),[32] the amine was allowed to react with *o*-nosyl chloride (→**15**) followed by glycosylation with 2,3-dichlorobenzyl alcohol to yield intermediate **16**. Acylation by modified Staudinger conditions[33] and deprotection yielded the acyl derivatives **17a–c**. The low yields in this particular reaction can be explained by the formation of side products due to acetate migration. In addition, because of the small scale, purification, especially removal of PPh_3=O, was extremely difficult. Interestingly, lithium hydroxide in water/tetrahydrofuran had to be used for deprotection, because treatment with 10% aqueous sodium hydroxide resulted in loss of the nitro substituent. To avoid acetate migration, the 9-amino derivative **19** was obtained by deprotection of **16** (→**18**) followed by azide reduction. Upon alkylation of the amino group by reductive amination, reduction of the nitro group could not be avoided. However, alkylation of the primary amine with 1-(bromomethyl)-4-phenylbenzene in the presence of potassium carbonate furnished sialoside **20**, although only in a modest yield.

Surface plasmon resonance (SPR)

The compound library **9a–k** (Table 1) and the newly synthesized CD22 ligands **13a,b**, **17a–c**, and **20** were evaluated by SPR (Table 2). Here, *h*$CD22_{d1-3}$–Fc[34] was immobilized on a protein A surface, which had been covalently attached to the chip by amino coupling. In the reference cell, only protein A was immobilized to compensate for unspecific binding to the matrix. Dilution series of the compounds were prepared either in pure HBS-EP buffer (**9a–k**, **13a,b**) or in the same buffer additionally containing 5% DMSO (**17a–c**, **20**). Finally, the sensorgrams were fitted according to a 1:1 binding model.

Ligand **13a** showed a binding affinity in the submicromolar range (Entry 13). With the 2,3-dichlorobenzyl aglycone (→**13b**), a gain in affinity by a factor of eight was observed (Entry 14). Modifications at the 9-position caused a gain in affinity for 4-(4-hydroxyphenyl)benzamide **17b** (Entry 16), equal affinity for 4-(pyridin-4-yl)benzamide **17c** (Entry 17), and a loss in affinity for amine **20** (Entry 18). Surprisingly, **17a** (Entry 15) showed no affinity, neither in the SPR experiments, nor in the isothermal titration calorimetry (ITC) assay (Table 2), although the best substituents identified so far for the 2- (2,3-dichlorobenzyl) and the 5-positions (*o*-nosyl) were present. Regarding selectivity, **17b** exhibits a 50-fold higher affinity for CD22 than for MAG (K_D = 3.0 μM). This might be largely due to the biphenyl moiety at the 9-position, which has previously been shown to decrease the affinity for MAG.[20,28,35]

For the ligands **13b**, **17b**, and **17c** a clear prolongation of the residence time of the protein–ligand complex was observed (Table 3, Figure 2). Potential advantages of a prolonged

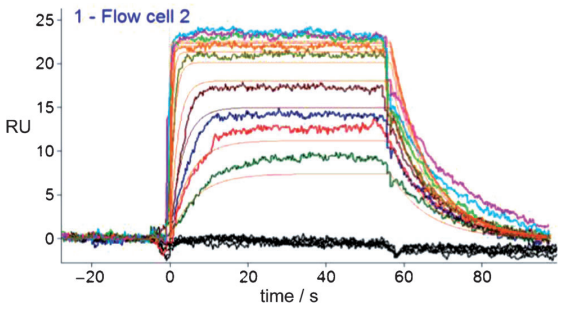
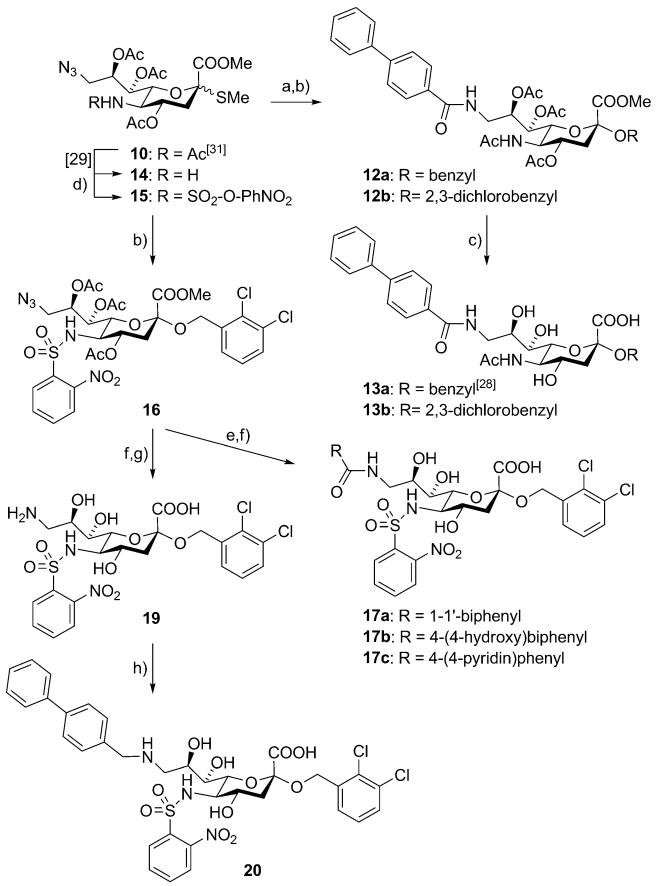


Figure 2. Sensorgram of **17b** at 25 °C measured in SPR; RU = response units.



Scheme 1. *Reagents and conditions:* a) 4-phenylbenzoyl chloride, PPh_3, DCE (77%); b) ROH, NIS, TfOH, CH_3CN [**12a**: 70%, **12b**: 50%, **16**: 76%]; c) 10% aq NaOH [**13a**: 51%, **13b**: 25%]; d) *o*-nosyl-Cl, NEt_3, DMAP, CH_2Cl_2 (78%); e) RCOCl, PPh_3, DCE; f) LiOH, H_2O, THF [**17a–c**: 6–19% for two steps, →**18**: 52%]; g) PPh_3, H_2O, NEt_3, 50 °C (13%); h) 1-(bromomethyl)-4-phenylbenzene, K_2CO_3, THF (12%).

of the ligand–CD22 interaction, but also the pharmacokinetic properties of the best representatives.

Starting from sialyl donor **10**,[31] test compound **13b** was obtained according to published procedures.[28,29] The *o*-nosyl derivatives were synthesized according to a different approach (Scheme 1). After cleavage of the *N*-acetate (→**14**),[32] the amine was allowed to react with *o*-nosyl chloride (→**15**) followed by glycosylation with 2,3-dichlorobenzyl alcohol to yield intermediate **16**. Acylation by modified Staudinger conditions[33] and deprotection yielded the acyl derivatives **17a–c**. The low yields in this particular reaction can be explained by the formation of side products due to acetate migration. In addition, because of the small scale, purification, especially removal of PPh_3=O, was extremely difficult. Interestingly, lithium hydroxide in water/tetrahydrofuran had to be used for deprotection, because treatment with 10% aqueous sodium hydroxide resulted in loss of the nitro substituent. To avoid acetate migration, the 9-amino derivative **19** was obtained by deprotection of **16** (→**18**) followed by azide reduction. Upon alkylation of the amino group by reductive amination, reduction of the nitro group could not be avoided. However, alkylation of the primary amine with 1-(bromomethyl)-4-phenylbenzene in the presence of potassium carbonate furnished sialoside **20**, although only in a modest yield.

Surface plasmon resonance (SPR)

The compound library **9a–k** (Table 1) and the newly synthesized CD22 ligands **13a,b**, **17a–c**, and **20** were evaluated by SPR (Table 2). Here, *h*$CD22_{d1-3}$–Fc[34] was immobilized on a protein A surface, which had been covalently attached to the chip by amino coupling. In the reference cell, only protein A was immobilized to compensate for unspecific binding to the matrix. Dilution series of the compounds were prepared either in pure HBS-EP buffer (**9a–k**, **13a,b**) or in the same buffer additionally containing 5% DMSO (**17a–c**, **20**). Finally, the sensorgrams were fitted according to a 1:1 binding model.

Ligand **13a** showed a binding affinity in the submicromolar range (Entry 13). With the 2,3-dichlorobenzyl aglycone (→**13b**), a gain in affinity by a factor of eight was observed (Entry 14). Modifications at the 9-position caused a gain in affinity for 4-(4-hydroxyphenyl)benzamide **17b** (Entry 16), equal affinity for 4-(pyridin-4-yl)benzamide **17c** (Entry 17), and a loss in affinity for amine **20** (Entry 18). Surprisingly, **17a** (Entry 15) showed no affinity, neither in the SPR experiments, nor in the isothermal titration calorimetry (ITC) assay (Table 2), although the best substituents identified so far for the 2- (2,3-dichlorobenzyl) and the 5-positions (*o*-nosyl) were present. Regarding selectivity, **17b** exhibits a 50-fold higher affinity for CD22 than for MAG (K_D = 3.0 μM). This might be largely due to the biphenyl moiety at the 9-position, which has previously been shown to decrease the affinity for MAG.[20,28,35]

For the ligands **13b**, **17b**, and **17c** a clear prolongation of the residence time of the protein–ligand complex was observed (Table 3, Figure 2). Potential advantages of a prolonged

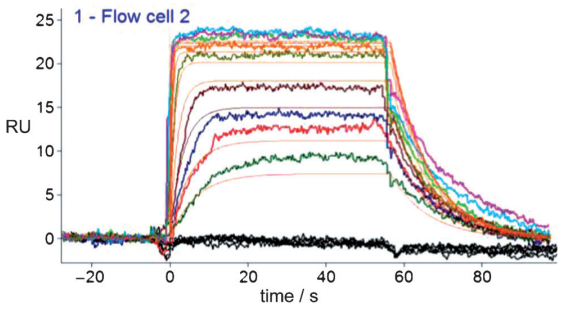
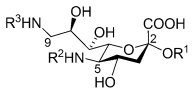
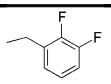
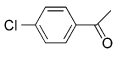
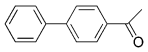
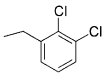
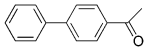
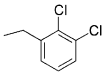
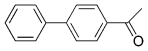
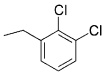
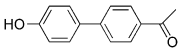
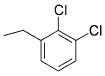
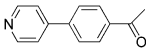
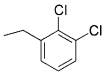
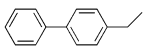


Figure 2. Sensorgram of **17b** at 25 °C measured in SPR; RU = response units.

Table 2. Affinity data for CD22 ligands as determined by SPR and ITC experiments.



Entry	Compd	R^1	R^2	R^3	K_D [nM] SPR	K_D [nM] ITC
12	**9k**				110	131
13	**13a**	Bn	Ac		800	751 ± 224
14	**13b**		Ac		100	82 ± 19
15	**17a**				NB[a]	NB[a]
16	**17b**				60	80 ± 9
17	**17c**				110	152 ± 43
18	**20**				500	ND[b]

[a] No binding. [b] Not determined.

Table 3. Kinetic properties of **9k**, **13b**, and **17b,c**.

Entry	Compd	K_D [nM]	k_{on} [10^6 $M^{-1}s^{-1}$]	k_{off} [s^{-1}]	$t_{1/2}$ [s][a]
19	**9k**	130	2.1	0.27	2.6
20	**13b**	100	1.1	0.13	5.3
21	**17b**	60	1.1	0.08	8.7
22	**17c**	110	1.3	0.16	4.3

[a] $t_{1/2} = \ln 2/k_{off}$.

residence time are extended duration of the pharmacological effect and target selectivity.[36] Additionally, a therapeutic effect can be reached with a lower dose. As the half-life ($t_{1/2}$) of carbohydrate–protein complexes is typically very short (< 1 s),[37,38] this is clearly an improved property of these new CD22 ligands, although there is still a need for further improvement. The extended $t_{1/2}$ of the ligand–CD22 complex[39] might be due to the improved lipophilicity of the ligands (see log*D* values in Table 5 below).

Isothermal titration calorimetry (ITC)

The binding affinities measured by SPR and ITC experiments (Table 2) are in good agreement with each other. The thermodynamic fingerprint of a ligand describes enthalpy and entropy contributions of the interaction with its target and therefore provides characteristic insight into the binding process. ITC measurements for the ligands **9k**, **13a,b**, and **17a–c** were performed at 25 °C using *h*CD22$_{d1-3}$–Fc[34] (Table 4) and confirmed the behavior typically observed for carbohydrate–lectin interactions,[40–43] namely an enthalpy-driven binding. Less common are entropy-driven carbohydrate–lectin interactions, such as heparin binding to the agrin-G3 domain[42] or the interaction of di- and trisaccharides with calreticulin.[43]

The interaction of the parent compound **9k** is dominated by a large enthalpic contribution, which is, however, decreased by substantial entropy costs (Entry 23). The approximate sixfold drop in affinity observed for **13a** results from a large increase in entropy costs, which is only partially compensated by an improved enthalpy contribution (Entry 24). A possible explanation for the increased entropy term is a substantial conformational change caused by the spatial demands of the biphenyl substituent. Replacement of the benzyl aglycone in **13a** with the 2,3-dichlorobenzyl group (→**13b**, Entry 25) gives a further increase in enthalpy, whereas the entropy term remains stable.

Surprisingly, **17a** (Entry 26) does not bind to CD22, although the *o*-nosyl substituent does not prohibit binding of **9k**. As reported by Kiso and colleagues,[19] intramolecular attraction between benzyl at the 2-position and biphenyl at the 9-position can lead to a hydrophobic collapse. The introduction of an *o*-nosyl group at the 5-position may further support this process, abolishing the binding of **17a**. This hypothesis is also supported by the pharmacokinetic properties of **17a** because it is the only compound that was retained in an artificial membrane

Table 4. Thermodynamic parameters as determined at 25 °C by ITC.

Entry	Compd	*N*	K_D [nM]	ΔG° [kJ mol^{-1}]	ΔH° [kJ mol^{-1}]	$-T\Delta S^\circ$ [kJ mol^{-1}]
23	**9k**	0.96	131	−39.3	−54.8	+15.5
24	**13a**	0.96 ± 0.04	751 ± 224	−35.1 ± 0.8	−73.4 ± 0.8	+38.3 ± 1.6
25	**13b**	0.94 ± 0.02	82 ± 19	−40.5 ± 0.6	−80.2 ± 3.4	+39.7 ± 3.6
26	**17a**	NB[a]	NB[a]	NB[a]	NB[a]	NB[a]
27	**17b**	1.07 ± 0.01	80 ± 9	−40.6 ± 0.4	−61.6 ± 1.1	+21.0 ± 0.7
28	**17c**	0.95 ± 0.02	152 ± 43	−39.0 ± 0.7	−83.4 ± 2.8	+44.4 ± 3.5

[a] No binding.

Table 5. Pharmacokinetic properties of CD22 ligands.

Entry	Compd	log $D_{7.4}$	PAMPA log P_e	PAMPA [% Mm]	PPB [%]
29	**13a**	0.91	NP[a]	NR[b]	97
30	**13b**	2.24	−9.8	NR[b]	98
31	**17a**	3.25	−8.7	pH 5.0: 24% pH 6.2: 14% pH 7.4: NR[b]	>99
32	**17b**	3.12	NP[a]	NR[b]	>99
33	**17c**	2.08	−8.9	NR[b]	>99

[a] No permeation. [b] No retention.

(PAMPA,[44] see Table 5, Entry 31). Interestingly, compound **20**, in which the amide linker of **17a** is replaced by an amine linker, again exhibited nanomolar binding affinity in SPR (Table 2, Entry 18).

Introduction of 4-(4-hydroxyphenyl)benzamide (→**17b**, Entry 27) resulted in a ligand with an affinity similar to that of compound **13b**, although the ΔH° and $T\Delta S^\circ$ terms substantially change; they both differ by 19 kJ mol^{-1}, but with opposite signs. Introduction of a *para*-hydroxy substituent caused a favorable enthalpy change similar to an observation reported by Kiso et al.[20] Interestingly, when the terminal phenol (→**17b**) was replaced by pyridine (→**17c**, Entry 28), a substantially improved ΔH° value (−22 kJ mol^{-1}) was observed, which, however, is overcompensated by a loss of entropy leading to an overall twofold decrease in affinity. Clearly, the *o*-nosyl substituent elicits a reorientation of the ligand in the binding site, or leads to a remarkable change in desolvation energies. As a consequence, a dramatic change in the thermodynamic fingerprint results. An enthalpy–entropy plot (Figure 3) reveals a linear relationship with a high correlation coefficient ($R^2 = 0.97$), indicating enthalpy–entropy compensation, a property often reported for carbohydrate–lectin interactions.[40,45] The slope of −1 denotes that the enthalpic gain is completely compensated by an entropic penalty.

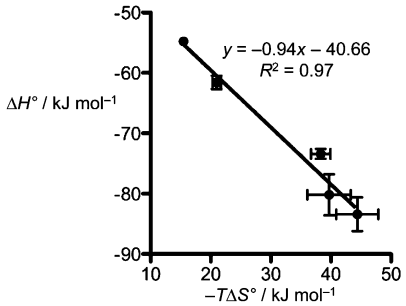


Figure 3. Enthalpy–entropy compensation plot: correlation of the change in enthalpy (ΔH°) and the change in entropy ($-T\Delta S^\circ$) of CD22 ligands interacting with hCD22$_{d1-3}$–Fc.[34]

Pharmacokinetics

As CD22 is found exclusively on B-cells, oral or intravenous applications must be considered. To elucidate the potential for oral availability, log D values were determined, as they are generally considered a rough indicator of a given compound's membrane permeation behavior,[46,47] although restrictions apply.[48] The log D values of compounds **13a**,**b** and **17a**–**c** were determined, and are in the range of 0.91–3.25 (Table 5), which indicates possible membrane permeability. However, determination of log P_e with the parallel artificial membrane permeability assay (PAMPA)[44] showed that **13a** and **17b** do not permeate at all, and **13b** and **17a**,**c** only to a very limited extent. Furthermore, retention within the membrane (PAMPA % Mm) could be excluded, except for **17a**. Here, at pH 5, 24% of the total amount of compound was retained in the membrane. The different results in the PAMPA for other ligands despite similar log D values might be caused by other molecular descriptors. For example, the log D values of **17a** and **17b** are in the same range, but their nonpolar surface areas and hydrogen bonding capacity differ. The former are generally observed to favor partition, whereas the latter seems to hinder it.[49] Due to these findings, no oral absorption based on passive diffusion can be expected for the presented CD22 ligands, as log P_e values below −6.7 indicate a very poor permeation through membranes. Therefore, if no active transport processes are involved, oral administration seems unrealistic.

Finally, plasma protein binding (PPB) of our CD22 ligands turned out to be very high. As CD22 is located on the surface of B-cells which circulate in the blood, high PPB might be beneficial, as suggested by Urien et al. for cetirizine, as an example.[50] Moreover, side effects due to distribution in various tissues of the body might be decreased as a consequence of both the high extent of PPB and the compound's inability to cross membranes. An additional positive aspect might be prolongation of plasma $t_{1/2}$, resulting from the fact that only free ligands can be metabolized and excreted. Nevertheless, the amount of unbound ligand, and consequently its distribution and clearance, depends not only on the extent of binding at equilibrium but also on the rates of association and dissociation (k_{on} and k_{off})[51] as well as tissue distribution and binding. The latter seems negligible, as PAMPA results do not indicate membrane permeation. The kinetic behavior of the ligands with respect to plasma proteins could be assessed by SPR and could provide valuable additional information.[52]

Conclusions

A series of high-affinity CD22 ligands was synthesized and investigated with regard to their biological and pharmacokinetic properties. The best ligand, sialoside **17b**, contains a dichlorobenzyl substituent at the anomeric position, an *ortho*-nitroben-

zylsulfonamide at the 5-position, and a 4′-hydroxy-4-biphenylcarboxamide at the 9-position. In addition to nanomolar affinity, ligand **17b** exhibits a prolonged residence time of the protein–ligand complex, which is atypical for carbohydrate–lectin interactions.[38] With respect to its pharmacokinetic properties, **17b** is not expected to be orally available based on PAMPA data, and therefore intravenous administration would need to be considered for an in vivo validation. However, the low permeation may be improved by a prodrug approach[47] or by replacing the carboxylate with a bioisostere.[53] Finally, **17b** shows extended plasma protein binding, which might be beneficial concerning various aspects such as pharmacological effect, plasma half-life, or side effects.

Experimental Section

Chemistry

NMR spectra were recorded on a Bruker Avance DMX-500 spectrometer (500 MHz). Assignment of ^{1}H and ^{13}C NMR spectra was performed by using 2D methods (COSY, HSQC, TOCSY). Chemical shifts are expressed in ppm using residual $CHCl_3$, CHD_2OD and HDO as references. Optical rotations were measured with PerkinElmer Polarimeters 241 and 341. MS analyses were carried out with a Waters Micromass ZQ Detector system. The spectra were recorded in positive or negative ESI mode. HPLC–HRMS analyses were carried out with an Agilent 1100 instrument equipped with a photodiode array detector and a Micromass QTOF I equipped with a 4 GHz digital time converter. All target compounds exhibited a purity of $\geq$95%. Reactions were monitored by TLC using glass plates coated with silica gel 60 F_{254} (Merck) and visualized by using UV light and/or by charring with a molybdate solution (0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in 10% aq H_2SO_4). Column chromatography was performed on silica gel (Uetikon, 40–60 mesh). CH_3OH was dried by holding at reflux with NaOMe and distilled immediately before use. THF was distilled over Na immediately before use. CH_2Cl_2, dichloroethane (DCE), CH_3CN, toluene, and benzene were dried by filtration over Al_2O_3 (Fluka, type 5016 A basic). Molecular sieves (3 Å) were activated under vacuum at 500 °C for 2 h immediately before use. Synthetic procedures for compounds **10**,[31] **12a**, **13a**,[28] **14**, and **15**[29] were reported earlier.

Methyl [methyl 5-acetamido-4,7,8-tri-*O*-acetyl-3,5,9-trideoxy-9-(4-phenylbenzamido)-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate (11): Compound **10** (250 mg, 0.50 mmol, 1.0 equiv) was dissolved in dry DCE (10 mL). 4-Phenylbenzoyl chloride (432 mg, 2.00 mmol, 4.0 equiv) and PPh_3 (290 mg, 1.10 mmol, 2.2 equiv) were added, and the reaction mixture was stirred at room temperature for 24 h. After dilution with CH_2Cl_2 (10 mL), the organic layer was washed with satd aq $NaHCO_3$ (3×5 mL) and H_2O (5 mL), dried over Na_2SO_4 and filtered. Afterward, the solvents were removed under reduced pressure and the crude product was purified by chromatography on silica gel (0.5% gradient of CH_3OH in CH_2Cl_2) to yield **11** (251 mg, 77%) as an oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.90 (s, 3H, OAc), 1.97 (s, 3H, SMe), 2.03, 2.07 (2 s, 6H, 2 OAc), 2.20 (dd, J = 11.8, 13.8 Hz, 1H, H-3a), 2.24 (s, 3H, NHAc), 2.56 (dd, J = 4.9, 13.8 Hz, 1H, H-3b), 3.05 (ddd, J = 0.5, 3.5, 15.3 Hz, 1H, H-9a), 3.82 (s, 3H, OMe), 4.16 (q, J = 10.3 Hz, 1H, H-5), 4.28 (dd, J = 1.9, 10.6 Hz, 1H, H-6), 4.50 (ddd, J = 3.9, 8.6, 15.0 Hz, 1H, H-9b), 5.16 (dt, J = 3.5, 7.3 Hz, 1H, H-8), 5.20–5.33 (m, 2H, H-4, H-7), 5.43 (d, J = 10.1 Hz, 1H, 5-NH), 7.22 (dd, J = 4.3, 8.5 Hz, 1H, 9-NH), 7.36–7.43 (m, 1H, CH_{ar}), 7.47 (dd, J = 4.8, 10.3 Hz, 2H, CH_{ar}), 7.54–7.63 (m, 2H, CH_{ar}), 7.64–7.72 (m, 2H, CH_{ar}), 7.90 ppm (d, J = 8.5 Hz, 2H, CH_{ar}); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 11.4 (SMe), 21.0, 21.3 (3C, 3 OAc), 23.3 (NHAc), 37.3 (C3), 38.1 (C9), 49.9 (C5), 53.1 (OMe), 68.4 (C7), 69.4 (C4), 70.7 (C8), 71.0 (C6), 84.9 (C2), 127.3, 127.4, 127.7, 128.2, 129.1, 133.0, 140.1, 144.5 (12C, C_{ar}), 167.3, 168.2, 170.4, 170.5, 171.2, 172.2 ppm (6 CO); MS (ESI) m/z calcd for $C_{32}H_{38}N_2O_{11}S$ $[M+Na]^+$: 681.20, found: 681.28.

Methyl [2,3-dichlorobenzyl 5-acetamido-4,7,8-tri-*O*-acetyl-3,5,9-trideoxy-9-(4-phenylbenzamido)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (12b): Compound **11** (50 mg, 7.5 μmol, 1.0 equiv) was dissolved in dry CH_3CN (2.0 mL). 2,3-Dichlorobenzyl alcohol (40 mg, 23 μmol, 3.0 equiv) and powdered MS (3 Å) were added. The mixture was stirred at room temperature for 1.5 h. The suspension was then cooled to −40 °C and subsequently treated with *N*-iodosuccinimide (NIS; 27 mg, 12 μmol, 1.6 equiv) and triflic acid (5.3 μL, 6 μmol in 0.2 mL CH_3CN, 0.8 equiv). After 30 min, the reaction mixture was warmed to −30 °C and stirring was continued for 16 h. The mixture was allowed to warm to room temperature, stirred for another 2 h and filtered through a pad of Celite. The Celite was washed with CH_2Cl_2 (5 mL) and the filtrate was subsequently washed with 20% aq $Na_2S_2O_3$ (1 mL) and satd aq $NaHCO_3$ (3×2 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (0.5% gradient of *i*PrOH in petroleum ether (PE)/CH_2Cl_2 8:4) to yield **12b** (30 mg, 50%) as an oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.89 (s, 6H, NHAc, OAc), 2.01–2.06 (m, 4H, H-3a, OAc), 2.15 (s, 3H, OAc), 2.57 (dd, J = 4.1, 13.4 Hz, 1H, H-3b), 3.49 (dd, J = 5.6, 12.2 Hz, 1H, H-9a), 3.79 (s, 3H, OMe), 3.83 (d, J = 12.4 Hz, 1H, H-9b), 4.09 (t, J = 10.5 Hz, 1H, H-5), 4.15 (m, 1H, H-6), 4.58 (A of AB, J = 13.7 Hz, 1H, CH_2Ar), 4.83–4.98 (m, 2H, H-4, CH_2Ar), 5.33–5.40 (m, 2H, H-7, H-8), 7.21 (t, J = 7.5 Hz, 1H, CH_{ar}), 7.23–7.31 (m, 4H, CH_{ar}), 7.34–7.50 (m, 6H, CH_{ar}), 7.56–7.69 ppm (m, 1H, CH_{ar}); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.8, 20.9, 21.1 (3 OAc), 23.3 (NHAc), 37.8 (C3), 43.2 (C9), 49.5 (C5), 53.0 (OMe), 64.3 (CH_2Ar), 68.3 (C7), 68.9 (C4), 70.7 (C8), 73.0 (C6), 98.8 (C2), 126.2, 127.2, 127.3, 127.5, 129.4, 129.6, 137.3 (18C, C_{ar}), 168.1, 170.2, 170.4, 170.7, 171.0, 171.2 ppm (6 CO); MS (ESI) m/z calcd for $C_{38}H_{40}Cl_2N_2O_{12}$ $[M+Na]^+$: 809.18, found: 809.28.

Sodium [2,3-dichlorobenzyl 5-acetamido-3,5,9-trideoxy-9-(4-phenylbenzamido)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (13b): Compound **12b** (30 mg, 38 μmol) was treated with 10% aq NaOH (0.4 mL) in CH_3OH (2 mL). The crude product was purified by LC–MS to yield **13b** as a white solid (6.3 mg, 25%). $[\alpha]_D^{20}$ −21.8 (c = 0.33, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD): δ = 1.73 (t, J = 11.0 Hz, 1H, H-3a), 2.00 (s, 3H, NHAc), 2.93 (d, J = 11.4 Hz, 1H, H-3b), 3.46 (d, J = 9.0 Hz, 1H, H-7), 3.53 (m, 1H, H-9a), 3.68 (d, J = 9.0 Hz, 1H, H-6), 3.71–3.85 (m, 3H, H-4, H-5, H-9b), 4.04 (t, J = 7.7 Hz, 1H, H-8), 4.73 (A, B of AB, J = 13.4 Hz, 2H, CH_2Ar), 7.26 (t, J = 7.9 Hz, 1H, CH_{ar}), 7.39 (t, J = 6.6 Hz, 2H, CH_{ar}), 7.46 (t, J = 7.4 Hz, 2H, CH_{ar}), 7.57 (d, J = 7.7 Hz, 1H, CH_{ar}), 7.67 (d, J = 7.4 Hz, 2H, CH_{ar}), 7.72 (d, J = 7.8 Hz, 2H, CH_{ar}), 7.91 (d, J = 7.9 Hz, 2H, CH_{ar}), 8.22 (d, J = 6.5 Hz, 1H, 5-NH), 8.30 ppm (s, 1H, 9-NH); ^{13}C NMR (125 MHz, CD_3OD): δ = 22.6 (NHAc), 42.5 (C3), 44.5 (C9), 54.1 (C5), 64.6 (CH_2Ar), 69.6 (C4), 71.2 (C8), 72.5 (C7), 74.5 (C6), 101.4 (C2), 127.9, 128.0, 128.2, 128.5, 128.6, 129.0, 129.1, 130.0, 130.1, 140.3, 145.6 (18C, C_{ar}), 175.5 ppm (3C, 3 CO); HRMS (ESI) m/z calcd for $C_{31}H_{32}Cl_2N_2O_9$ $[M+Na]^+$: 669.1384, found: 669.1384.

Methyl [methyl 4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-5-(2-nitrophenylsulfonamido)-2-thio-D-glycero-α-D-galacto-2-nonulopyranosid]onate (15): Nosyl chloride (105 mg, 0.47 mmol), NEt_3 (34.0 μL, 48.0 mg, 0.47 mmol), and DMAP (10.0 mg, 0.08 mmol)

were added successively to a solution of **14** (73.0 mg, 0.16 mmol) in dry CH_2Cl_2 (3 mL) at 0 °C. The reaction mixture was stirred at room temperature overnight and then washed with satd aq $NaHCO_3$ (2×5 mL) and H_2O (5 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel (PE/EtOAc 1:1→1:2) to yield **15** (81 mg, 78%) as an oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.85 (m, 1H, H-3a), 2.02 (s, 3H, SMe), 2.10, 2.13, 2.21 (3 s, 9H, 3 OAc), 2.80 (m, 1H, H-3b), 3.32 (dd, J = 6.2, 13.4 Hz, 1H, H-9a), 3.57 (dd, J = 3.2, 13.4 Hz, 1H, H-9b), 3.80 (m, 1H, H-5), 3.82 (s, 3H, OMe), 3.91 (d, J = 10.5 Hz, 1H, H-6), 4.97 (td, J = 4.7, 11.4 Hz, 1H, H-4), 5.30 (m, 2H, H-7, H-8), 5.75 (d, J = 9.4 Hz, 1H, NH), 7.70 (m, 2H, CH_{ar}), 7.90 (d, J = 7.9, 1H, CH_{ar}), 8.10 ppm (d, J = 6.5, 1H, CH_{ar}); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 12.1 (SMe), 20.5, 21.1, 21.1 (3 OAc), 38.1 (C3), 50.8 (C9), 53.2, 53.5 (C5, OMe), 68.8 (C7), 69.7 (C4), 70.4 (C8), 74.7 (C6), 82.8 (C2), 125.5, 130.4, 133.5, 135.5, 147.5 (6C, C_{ar}), 167.8, 170.4 ppm (4C, 4 CO); MS (ESI) *m/z* calcd for $C_{23}H_{29}N_5O_{13}S_2$ $[M-H]^-$: 646.12, found: 646.56.

Methyl [2,3-dichlorobenzyl 4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-5-(2-nitrophenylsulfonamido)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (16): Compound **15** (370 mg, 0.57 mmol, 1.0 equiv) was dissolved in dry CH_3CN (10 mL). Then, 2,3-dichlorobenzyl alcohol (302 mg, 1.71 mmol, 3.0 equiv) and powdered MS (3 Å) were added. The mixture was stirred at room temperature for 1.5 h. Afterward, the suspension was cooled to −40 °C and subsequently treated with NIS (205 mg, 0.92 mmol, 1.6 equiv) and triflic acid (40 μL, 0.4 mmol, 0.8 equiv). After 30 min, the reaction mixture was warmed to −30 °C, and stirring was continued for 16 h. The mixture was then warmed to room temperature, stirred for another 2 h and filtered through a pad of Celite. The Celite was washed with CH_2Cl_2 (15 mL), and the filtrate was subsequently washed with 20% aq $Na_2S_2O_3$ (5 mL), satd aq $NaHCO_3$ (3×5 mL), and H_2O (5 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel (0.5% gradient of *i*PrOH in PE/CH_2Cl_2 2:1) to yield **16** as a yellow solid (337 mg, 76%). $[\alpha]_D^{20}$ −43.7 (c = 0.46, CH_3OH); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.96 (m, 1H, H-3a), 2.02, 2.14, 2.22 (3 s, 9H, 3 OAc), 2.75 (m, 1H, H-3b), 3.31 (m, 1H, H-9a), 3.50 (m, 1H, H-9b), 3.78 (s, 3H, OMe), 3.88 (m, 1H, H-5), 4.15 (d, J = 10.6 Hz, 1H, H-6), 4.58, 4.66 (A, B of AB, J = 12.7, 2H, CH_2Ar), 4.96 (m, 1H, H-4), 5.24–5.32 (m, 2H, H-7, H-8), 5.60 (d, J = 9.3 Hz, 1H, 5-NH), 7.25 (m, 1H, CH_{ar}), 7.35–7.48 (m, 2H, CH_{ar}), 7.74 (dd, J = 4.5, 10.7 Hz, 1H, CH_{ar}), 7.77–7.86 (m, 2H, CH_{ar}), 8.19 ppm (m, 1H, CH_{ar}); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 20.5, 20.7, 21.0 (3 OAc), 38.2 (C3), 50.5 (C9), 51.0 (OMe), 52.9 (C5), 64.4 (CH_2Ar), 68.6 (C4), 69.7 (C7), 71.1 (C8), 76.7 (C6), 98.1 (C2), 123.7, 125.4, 126.4, 127.5, 127.7, 129.4, 132.4, 132.7, 134.1, 135.4, 149.5 (12C, C_{ar}), 168.6, 170.7, 170.9 ppm (4C, 4 CO); MS (ESI) *m/z* calcd for $C_{29}H_{31}Cl_2N_5O_{14}S$ $[M+Na]^+$: 798.08, found: 797.95.

General procedure A for the preparation of acid chlorides: The acid (0.15 mmol) was suspended in dry CH_2Cl_2 (1–2 mL) and cooled to 0 °C. Chloroenamine (0.17 mmol) was added dropwise, and the reaction was allowed to warm to room temperature. After 2 h, the initial precipitate was dissolved, and the acid chlorides were directly used without work-up and purification.

General procedure B for the synthesis of compounds 17a–c: Compound **16** (75 μmol) was added to the corresponding acid chloride (0.15 mmol), dissolved in dry CH_2Cl_2 (1 mL). PPh_3 (0.18 mmol) in dry CH_2Cl_2 (1.5 mL) was added after 5 min, and the solution was stirred at room temperature for 24 h. Then, the reaction mixture was diluted with CH_2Cl_2 (5 mL) and washed with satd aq $NaHCO_3$ (3×3 mL) and H_2O (3 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by chromatography on silica gel. Afterward, the acetylated intermediate was dissolved in THF/H_2O (2.5 mL, 4:1) and treated with LiOH (0.28 mmol) at room temperature for 4 h. After completion of the reaction, the crude product was purified by LC–MS to yield the final compound.

Sodium [2,3-dichlorobenzyl 3,5,9-trideoxy-5-(2-nitrophenylsulfonamido)-9-(4-phenylbenzamido)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (17a): Prepared from **16** (50 mg, 0.06 mmol) and 4-phenyl benzoic acid according to general procedures A and B. Yield: 10 mg (19%) as a white solid. $[\alpha]_D^{20}$ −1.8 (c = 0.04, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD): δ = 1.64 (t, J = 12.1 Hz, 1H, H-3a), 2.40 (dd, J = 3.7, 13.0 Hz, 1H, H-3b), 3.51 (m, 1H, H-9a), 3.59 (t, J = 10.0 Hz, 1H, H-5), 3.69–3.80 (m, 2H, H-7, H-9b), 3.98 (m, 1H, H-8), 4.05 (m, 1H, H-4), 4.12 (d, J = 10.5 Hz, 1H, H-6), 4.53, 4.85 (A, B of AB, J = 12.3 Hz, 2H, CH_2Ar), 7.29 (t, J = 8.3 Hz, 1H, CH_{ar}), 7.38 (t, J = 7.2 Hz, 1H, CH_{ar}), 7.42–7.49 (m, 3H, CH_{ar}), 7.64 (d, J = 7.7 Hz, 1H, CH_{ar}), 7.68 (d, J = 7.1 Hz, 3H, CH_{ar}), 7.73 (d, J = 7.6 Hz, 4H, CH_{ar}), 7.91 (d, J = 7.6 Hz, 2H, CH_{ar}), 8.18 (d, J = 7.6 Hz, 1H, CH_{ar}), 8.35 ppm (s, 1H, NH); ^{13}C NMR (125 MHz, CD_3OD): δ = 42.3 (C3), 45.7 (C9), 58.1 (C5), 63.9 (CH_2Ar), 68.4 (C4), 71.0 (C7), 71.2 (C8), 72.6 (C6), 99.3 (C2), 125.6, 125.9, 128.1, 128.2, 128.7, 129.0, 129.1, 129.2, 130.0, 130.3, 132.4, 133.5, 134.3, 136.5, 139.6, 141.3, 145.7, 149.0 (24C, C_{ar}), 171.0 ppm (2C, 2 CO); HRMS (ESI) *m/z* calcd for $C_{35}H_{32}Cl_2N_3NaO_{12}S$ $[M+Na]^+$: 834.0880, found: 834.0893.

Sodium [2,3-dichlorobenzyl 3,5,9-trideoxy-9-[4-(4-hydroxyphenyl)benzamido]-5-(2-nitrophenylsulfonamido)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (17b): Prepared from **16** (30 mg, 40 μmol) and 4-(4-hydroxyphenyl)benzoic acid according to general procedures A and B. Yield: 2.0 mg (6%) as a pale-yellow solid. $[\alpha]_D^{20}$ −88.0 (c = 0.01, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD): δ = 1.65 (t, J = 12.0 Hz, 1H, H-3a), 2.78 (dd, J = 3.6, 12.0 Hz, 1H, H-3b), 3.41–3.58 (m, 2H, H-5, H-9a), 3.67 (m, 1H, H-4), 3.70–3.79 (m, 2H, H-7, H-9b), 3.88 (d, J = 10.3 Hz, 1H, H-6), 3.97 (m, 1H, H-8), 4.64, 4.87 (A, B of AB, J = 12.6 Hz, 2H, CH_2Ar), 6.91 (d, J = 7.8 Hz, 2H, CH_{ar}), 7.26 (t, J = 7.7 Hz, 1H, CH_{ar}), 7.39 (d, J = 8.0 Hz, 1H, CH_{ar}), 7.47–7.60 (m, 3H, CH_{ar}), 7.67 (d, J = 7.8 Hz, 2H, CH_{ar}), 7.70–7.80 (m, 4H, CH_{ar}), 7.85 (d, J = 7.8 Hz, 2H, CH_{ar}), 8.16 (d, J = 7.3 Hz, 1H, NH), 8.23 ppm (m, 1H, NH); ^{13}C NMR (125 MHz, CD_3OD): δ = 42.6 (C3), 43.8 (C9), 49.5 (C5), 64.7 (CH_2Ar), 70.2, 71.2, 71.9 (C4, C7, C8), 74.0 (C6), 116.8, 125.8, 127.3, 128.6, 128.7, 128.9, 129.2, 130.1, 132.1, 132.5, 133.3, 133.7, 134.6, 135.8 (24C, C_{ar}), 170.5 ppm (2C, 2 CO); HRMS (ESI) *m/z* calcd for $C_{35}H_{33}Cl_2N_3O_{13}S$ $[M+Na]^+$: 828.1009, found: 828.1009.

Sodium [2,3-dichlorobenzyl 3,5,9-trideoxy-5-(2-nitrophenylsulfonamido)-9-[4-(pyridin-4-yl)benzamido]-D-glycero-α-D-galacto-2-nonulopyranosid]onate (17c): Prepared from **16** (58 mg, 70 μmol) and 4-(pyridin-4-yl)benzoic acid according to general procedures A and B. Yield: 8.0 mg (13%) as a pale-yellow solid. $[\alpha]_D^{20}$ −45.0 (c = 0.32, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD): δ = 1.71 (t, J = 12.1 Hz, 1H, H-3a), 2.72 (dd, J = 4.3, 12.3 Hz, 1H, H-3b), 3.51 (t, J = 10.0 Hz, 1H, H-5), 3.57 (dd, J = 6.3, 13.7 Hz, 1H, H-9a), 3.69 (td, J = 4.3, 11.3 Hz, 1H, H-4), 3.76–3.86 (m, 2H, H-7, H-9b), 3.91 (d, J = 10.3 Hz, 1H, H-6), 4.02 (t, J = 7.4 Hz, 1H, H-8), 4.69, 4.92 (A, B of AB, J = 13.2 Hz, 2H, CH_2Ar), 7.26 (t, J = 7.8 Hz, 1H, CH_{ar}), 7.40 (d, J = 8.0 Hz, 1H, CH_{ar}), 7.49 (d, J = 7.7 Hz, 1H, CH_{ar}), 7.76 (t, J = 6.2 Hz, 2H, CH_{ar}), 7.81 (m, 1H, CH_{ar}), 7.86–7.93 (m, 5H, CH_{ar}), 7.99 (d, J = 7.8 Hz, 2H, CH_{ar}), 8.17 (d, J = 7.0 Hz, 1H, NH), 8.67 ppm (d, J = 5.1 Hz, 2H, CH_{ar}); ^{13}C NMR (125 MHz, CD_3OD): δ = 42.3 (C3), 44.4 (C9), 57.9 (C5), 64.7 (CH_2Ar), 69.8 (C4), 71.2 (C7), 71.7 (C8), 74.5 (C6), 100.9 (C2), 123.9, 125.9, 128.5, 128.6, 128.7, 129.5, 130.4, 132.3, 133.5, 133.7, 134.6, 136.2, 136.9, 139.6, 141.2, 148.9, 151.4 (23C, C_{ar}), 170.0, 172.6 ppm

(2 CO); HRMS (ESI) *m/z* calcd for $C_{34}H_{32}Cl_2N_4O_{12}S$ $[M+H]^+$: 791.1193, found: 791.1194.

Sodium [2,3-dichlorobenzyl 9-azido-3,5,9-trideoxy-5-(2-nitrophenylsulfonamido)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (18): A solution of **16** (310 mg, 0.4 mmol) in THF/H_2O (6 mL:2.5 mL) was allowed to react with LiOH (78 mg, 3.2 mmol) at room temperature for 4 h. The crude product was purified by LC–MS to yield **18** (107 mg, 52%) as an oil. ^{1}H NMR (500 MHz, CD_3OD): $\delta=1.68$ (t, $J=$ 12.2 Hz, 1H, H-3a), 2.71 (dd, $J=4.8$, 12.5 Hz, 1H, H-3b), 3.31 (m, 1H, H-9a), 3.45 (m, 1H, H-5), 3.53 (dd, $J=2.4$, 12.8 Hz, 1H, H-9b), 3.67 (ddd, $J=4.8$, 9.9, 11.8 Hz, 1H, H-4), 3.81 (dd, $J=1.5$, 8.9 Hz, 1H, H-7), 3.86 (dd, $J=1.5$, 10.5 Hz, 1H, H-6), 3.95 (ddd, $J=2.4$, 6.6, 8.9 Hz, 1H, H-8), 4.70, 4.92 (A, B of AB, $J=13.3$ Hz, 2H, CH_2Ar), 7.27 (t, $J=$ 7.9 Hz, 1H, CH_{ar}), 7.44 (dd, $J=1.4$, 8.0 Hz, 1H, CH_{ar}), 7.50 (d, $J=$ 7.7 Hz, 1H, CH_{ar}), 7.70–7.81 (m, 2H, CH_{ar}), 7.88 (m, 1H, CH_{ar}), 8.16 ppm (dd, $J=3.3$, 6.0 Hz, 1H, CH_{ar}); ^{13}C NMR (125 MHz, CD_3OD): $\delta=42.4$ (C3), 55.0 (C9), 57.9 (C5), 64.6 (CH_2Ar), 69.6 (C4), 70.5 (C7), 72.4 (C8), 74.5 (C6), 125.8, 128.5, 128.7, 130.3, 132.3, 133.5, 134.4 (12C, C_{ar}), 172.5 ppm (CO); MS (ESI) *m/z* calcd for $C_{22}H_{23}Cl_2N_5O_{11}S$ $[M-H]^-$: 634.04, found: 634.16.

Sodium [2,3-dichlorobenzyl 9-amino-3,5,9-trideoxy-5-(2-nitrophenylsulfonamido)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (19): PPh_3 (33 mg, 0.13 mmol, 1.6 equiv) and NEt_3 (1.2 μL, 12 μmol, 1.5 equiv) were successively added to a solution of **18** (50 mg, 8.0 μmol, 1.0 equiv) in dry THF (5 mL) at 0 °C. After 1 h, PPh_3 (66 mg, 0.26 mmol, 3.2 equiv) and H_2O (1.5 mL) were added, and stirring was continued at 50 °C for 4 h. After cooling to room temperature, the solvent was removed under reduced pressure, and the residue was purified by LC–MS to yield **19** (6.3 mg, 13%) as an oil. $[\alpha]_D^{20}$ −43.7 ($c=0.46$, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD): $\delta=1.60$ (t, $J=12.0$ Hz, 1H, H-3a), 2.79 (dd, $J=4.9$, 12.3 Hz, 1H, H-3b), 2.93 (dd, $J=9.3$, 12.7 Hz, 1H, H-9a), 3.34 (m, 1H, H-9b), 3.38 (t, $J=10.1$ Hz, 1H, H-5), 3.66 (m, 1H, H-4), 3.80–3.89 (m, 2H, H-6, H-7), 4.05 (m, 1H, H-8), 4.69, 4.91 (A, B of AB, $J=13.6$ Hz, 2H, CH_2Ar), 7.27 (t, $J=7.9$ Hz, 1H, CH_{ar}), 7.42 (d, $J=8.0$ Hz, 1H, CH_{ar}), 7.54 (d, $J=7.8$ Hz, 1H, CH_{ar}), 7.71–7.81 (m, 2H, CH_{ar}), 7.89 (dd, $J=$ 3.3, 6.0 Hz, 1H, CH_{ar}), 8.17 ppm (dt, $J=3.7$, 7.5 Hz, 1H, CH_{ar}); ^{13}C NMR (125 MHz, CD_3OD): $\delta=43.0$ (C3), 43.9 (C9), 57.9 (C5), 64.5 (CH_2Ar), 69.5 (C8), 70.0 (C4), 72.0 (C7), 74.1 (C6), 125.9, 128.3, 128.6, 130.0, 132.3, 133.4, 133.6, 134.5, 136.0, 140.2, 149.1 (12C, C*ar*), 173.9 ppm (CO); MS (ESI) *m/z* calcd for $C_{22}H_{25}Cl_2N_3O_{11}S$ $[M+H]^+$: 610.07, found: 610.12.

Sodium [2,3-dichlorobenzyl 3,5,9-trideoxy-5-(2-nitrophenylsulfonamido)-9-(4-phenylbenzylamine)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (20): Compound **19** (2.0 mg, 3.3 μmol, 1.0 equiv) was dissolved in THF (0.6 mL). 1-(Bromomethyl)-4-phenylbenzene (1.1 mg, 6.6 μmol, 2.0 equiv) and K_2CO_3 (1.1 mg, 8.0 μmol, 2.4 equiv) were added in portions. The reaction mixture was stirred at room temperature for 4 days. The crude product was purified by LC–MS to yield **20** as an oil (0.3 mg, 12%). $[\alpha]_D^{20}$ −102.3 ($c=0.01$, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD): $\delta=1.60$ (t, $J=11.8$ Hz, 1H, H-3a), 2.77 (m, 1H, H-3b), 2.98 (t, $J=10.9$ Hz, 1H, H-9a), 3.39–3.41 (m, 2H, H-5, H-9b), 3.66 (m, 1H, H-4), 3.76–3.90 (m, 2H, H-6, H-7), 4.10 (t, $J=8.7$ Hz, 1H, H-8), 4.19 (s, 2H, NCH_2), 4.72, 4.95 (A, B of AB, 2H, CH_2Ar), 7.26 (t, $J=7.5$ Hz, 1H, CH_{ar}), 7.36 (m, 1H, CH_{ar}), 7.38–7.48 (m, 3H, CH_{ar}), 7.54 (d, $J=7.4$ Hz, 2H, CH_{ar}), 7.63 (d, $J=7.3$ Hz, 2H, CH_{ar}), 7.69 (d, $J=7.3$ Hz, 2H, CH_{ar}), 7.76 (s, 2H, CH_{ar}), 7.87 (s, 1H, CH_{ar}), 8.17 (s, 1H, CH_{ar}), 8.55 ppm (s, 1H, CH_{ar}); ^{13}C NMR (125 MHz, CD_3OD): $\delta=41.6$ (C3), 47.3 (C9), 51.2 (NCH_2), 57.0 (C5), 63.6 (CH_2Ar), 68.5 (C8), 69.1 (C4), 70.5 (C7), 72.3 (C6), 123.9, 126.6, 126.7, 127.1, 127.5, 127.6, 128.3, 128.4, 129.7, 130.6, 132.3 ppm (24C, C_{ar}); HRMS (ESI) *m/z* calcd for $C_{35}H_{35}Cl_2N_3O_{11}S$ $[M+$ Na$]^+$: 798.1267, found: 798.1262.

Biological assays

Siglec–Fc proteins: Human $CD22_{d1-3}$–Fc from CHO-Lec1 and murine MAG_{d1-3}–Fc from CHO-Lec3.2.8.1 cell culture supernatants were affinity purified as described previously,[34] dialyzed against 10 mM phosphate buffer (pH 7.4), sterile filtered, and stored at 4 °C. The proteins were analyzed by an ELISA and binding assay with immobilized fetuin.[34]

Surface plasmon resonance (SPR): The SPR measurements were performed on a Biacore 3000 SPR-based optical biosensor (Biacore, GE Healthcare, Uppsala, Sweden). Sensor chips (CM5), immobilization kits, maintenance supply, and HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% *v/v* surfactant P20) were purchased from GE Healthcare. The carboxy groups on the CM5 chip were activated for 10 min with a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.1 M 3-(*N*,*N*-dimethylamino)propyl-*N*-ethylcarbodiimide (EDC) at a flow rate of 10 μL min^{-1}. For immobilizing protein A (P6031, Sigma), a solution of 30 μg mL^{-1} in acetate buffer was injected over the activated surface for 10 min at a flow rate of 10 μL min^{-1}. Protein A densities of ~4000 RU were achieved. Flow cells were blocked with a 10 min injection of 1 M ethanolamine, pH 8.0. For capturing, *h*$CD22_{d1-3}$–Fc or *m*MAG_{d1-3}–Fc solutions of 30–40 μg mL^{-1} concentration in NaOAc (pH 5.0) were injected at a flow rate of 5 μL min^{-1} for 3 min. The surface was equilibrated with HBS-EP buffer overnight at a flow rate of 5 μL min^{-1}, achieving densities of ~3000–4000 RU. Tenfold dilution series of antagonists were freshly prepared in HBS-EP. All binding experiments were conducted at 25 °C at a flow rate of 20 μL min^{-1}. The samples were injected for 1 min followed by 1 min dissociation. Each sample concentration was measured in duplicate. Double referencing was applied to correct for bulk effects and other systematic artifacts (subtraction of reference surface and blank injections). Data processing and equilibrium binding constant determinations were accomplished with Scrubber (BioLogic Software, Version 1.1g or 2.0c). Kinetic data were simultaneously fit using Scrubber 2.0c. For compounds with low solubility (→**17a**–**c**, **20**), 5% DMSO (for molecular biology, >99.9%, Fluka) was used in the buffer. The running buffer was 5% DMSO in HBS-EP. The surface was equilibrated at a flow of 5 μL min^{-1} until the baseline was stable. To eliminate the influence of DMSO on the signals, a calibration curve was recorded with DMSO concentrations of 4.7–5.1%. The DMSO calibration was accomplished directly in Scrubber 2.0c.

Isothermal titration calorimetry (ITC): ITC experiments were performed using a VP-ITC instrument from MicroCal Inc. (GE Healthcare, Northampton, UK). The measurements were performed at 25 °C. Injections of 5–10 μL ligand solutions were added from a computer-controlled syringe at an interval of 5 min into the sample cell solution containing *h*$CD22_{d1-3}$–Fc[34] (cell volume: 1.4523 mL) with stirring (307 rpm). For control experiments, identical ligand solutions were injected into buffer without protein. The heat released in this control experiment was subtracted from the experimental data. The assay buffer was HBS-E (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 5% DMSO). The concentration of *h*$CD22_{d1-3}$–Fc was 1.8–11.1 μM, calculated in terms of monomer, determined by HPLC–UV,[54] and the ligand concentration was 60–200 μM. The experimental data were fitted to a theoretical titration curve (one-site binding model) using Origin version 7 (GE Healthcare, Northampton, UK), with ΔH° (enthalpy change in kJ mol^{-1}), K_A (association constant in M^{-1}), and *N* (number of binding sites). The

quantity $c = K_A \cdot Mt_0$, where Mt_0 is the initial macromolecule concentration, is important in titration microcalorimetry. The experiment was performed at c values between 2 and 85. Thermodynamic parameters were calculated from Equation (1):

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = RT \ln K_A = -RT \ln K_D \quad (1)$$

for which ΔG°, ΔH°, and ΔS° are the changes in free energy, enthalpy, and entropy of binding, respectively. T is the absolute temperature, and $R = 8.314\ J\,mol^{-1}\,K^{-1}$.[55]

log $D_{7.4}$ determination: Triplicate measurements were performed for every compound at two ratios of octanol to buffer according to the expected log $D_{7.4}$ value. Equal amounts of phosphate buffer (0.1 M, pH 7.4) and octanol were mixed and shaken vigorously for 5 min. Upon separation of the two phases, the buffer phase was withdrawn and analytes were dissolved therein (10^{-5} M). Predefined volumes of octanol and analyte solutions were transferred to a PCR plate, which was thermo-sealed with aluminum foil and shaken (2 h, 1350 rpm, 25 °C on a Heidolph Titramax 100 plate shaker (Heidolph, Schwabach, Germany). The plate was then centrifuged at 2000 rpm (657 *g*) and 25 °C for 5 min, and buffer samples were withdrawn from each well. The analyte concentrations were determined by LC–MS, and log $D_{7.4}$ values were calculated. Values were accepted if the mean values of the two ratios did not differ by >0.1 units.

Parallel artificial membrane permeation assay (PAMPA): log P_e was determined in a 96-well format. For each compound, measurements were performed in quadruplicate at three pH values: 5.0, 6.2, and 7.4. Each well of a deep-well plate was filled with 650 μL System Solution (pIon, Woburn MA, USA, P/N 110151) at the according pH value. Samples (150 μL) were withdrawn from each well to determine the blank spectra by UV/Vis spectroscopy. Analyte was then added to the remaining System Solution to yield 50 μM solutions. To exclude precipitation, the optical density was measured at λ 650 nm, with 0.01 being the threshold value. Again, samples of 150 μL were withdrawn to determine the reference spectra. A further 200 μL were transferred to each well of the donor plate of the PAMPA sandwich. The filter membranes at the bottom of the acceptor plate were impregnated with 5 μL GIT-0 Lipid Solution (pIon, P/N 110669), and 200 μL Acceptor Sink Buffer (pIon, P/N 110139) were filled into each acceptor well. The sandwich was assembled, then placed in the GutBox, left undisturbed for 16 h, and then disassembled again followed by the transfer of 150 μL from each donor and acceptor well to UV plates. Quantification was performed by both UV/Vis spectroscopy (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) and LC–MS; log P_e values were calculated based on the LC–MS results and with the aid of the PAMPA Explorer Software (pIon, version 3.5).

Plasma protein binding (PPB): The dialysis membranes (HTDialysis LCC, Gales Ferry, USA; MWCO 12–14 kDa) were prepared according to the manufacturer's instructions. Human plasma (Biopredic, Rennes, France) was centrifuged (5800 rpm (4325 *g*), 25 °C, 10 min), the centrifugate (without floating plasma lipids) was adjusted to pH 7.5, and analyte was added to yield 10 μM solutions. Equal volumes (150 μL each) of phosphate buffer (0.1 M, pH 7.5) and analyte-containing plasma were transferred to the separated compartments of the assembled 96-well high-throughput dialysis block (HTDialysis). Measurements were performed in triplicate. The plate was covered with a sealing film and incubated (5 h, 37 °C). Buffer and plasma compartment were processed separately: 90 μL were withdrawn from the buffer compartment and 10 μL blank plasma were added, while 10 μL were withdrawn from the plasma compartment and 90 μL of blank buffer were added. After protein precipitation with 300 μL CH_3CN (4 °C) the solutions were mixed, centrifuged (3600 rpm (1666 *g*), 4 °C, 11 min) and 50 μL of the supernatant were withdrawn. Analyte concentrations were determined by LC–MS. The fraction unbound was calculated by dividing the concentration in the buffer compartment by the concentration in the plasma compartment (both concentrations adjusted for dilutions prior to analysis). The fraction bound was calculated by subtracting the fraction bound from 1. Values were accepted if the recovery of analyte was between 80 and 120%.

LC–MS measurements: Separation was performed on an Agilent 1100 Series HPLC instrument with a 1200 series autosampler, connected to an Agilent 6400 Series Triple Quadrupole mass spectrometer for quantification (Agilent Technologies, Santa Clara, CA, USA). Double-distilled H_2O with 0.1% formic acid (A) and CH_3CN with 0.1% formic acid (B) were used as solvents. The gradient was a follows: 0.1 min 95% A to 5% B; 1 min 5% A to 95% B; 1.2 min 95% A to 5% B. The total method duration was 4 min. For the separation, a Waters Atlantis T3 column (3 μm, 2.1×50 mm) was used (Waters, Milford, MA, USA). The column was heated at 60 °C, and the flow rate was set to 0.6 mL min^{-1}; 5 μL of analyte were injected per run. Quantification was performed with the MassHunter software (Agilent Technologies, Version B.01.04).

Acknowledgements

We thank the Swiss National Science Foundation (project 200020-120628) for their support of this project.

Keywords: carbohydrate–lectin interactions · CD22 · MAG · pharmacokinetics · surface plasmon resonance · thermodynamic fingerprint

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Received: August 25, 2011
Published online on ■■ ▮▮, 0000

What does it need to achieve selectivity for Sialoadhesin, CD22 and MAG?

The achievement of selectivity among Siglecs is still a challenge. In the article, literature-reported novel approaches are discussed.

Stefanie Mesch, Johanna Tokarzewska-Zadora, Hendrik Koliwer-Brandl, Daniel Strasser, Oliver Schwardt, Paul Crocker, Soerge Kelm, Beat Ernst.

Work performed by Stefanie Mesch:
Synthesis of antagonists, performance and evaluation of Biacore assay.

What does it need to achieve selectivity for Sialoadhesin, CD22 and MAG?

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Keywords: CD22, Myelin-associated glycoprotein (MAG), selectivity, Sialoadhesin, Siglecs, surface plasmon resaonance

Introduction. The process of drug development demands to selectively address the target of interest and is of major importance in order to avoid side effects. Unfortunately, proteins of the same family often show highly conserved binding sites, *e.g.* Siglecs, making selectivity to a challenge for medicinal chemists. Sialoadhesin and CD22 are members of the Siglec-family[1] and are expressed on the surface of macrophages[2] and B-cells,[3] respectively. Consequently there might be a need for selectivity, as they are both in the same physiological compartment. In contrast, MAG is found on the surface of neurite cells,[4] but as it shows similar binding behavior as Sialoadhesin,[5] it was also considered.

Siglecs are a subset of the immunoglobuline superfamily, binding to sialic acid by definition.[6] As a consequence, their binding sites are well conserved and selectivity of their physiological ligands originates from different linkages of residual carbohydrates to the terminal sialic acid.[6] Hence, Sialoadhesin and MAG prefer α2,6-linked sialic acid, whereas CD22 shows a clear preference for α2,3-linkages.[7]

However, oligosaccharides are not considered as good drug candidates due to their low binding affinities and high structural complexity, their high polarity and fast excretion.[8] Consequently, introducing modifications at the sialic acid core, being only tolerated by one specific Siglec or combination of several substituents, might be a valuable approach for the achievement of selectivity. It has been suggested, that substitution in 5-position could be used to induce selectivity between the Siglec-members.[9] Hence, Kelm *et al.* showed that Gc5Ac is binding to CD22 but not to Sialoadhesin and MAG.[5] Furthermore, halogenated acetates in 5-position resulted in an increased binding affinity of MAG but not of Sialoadhesin.[5]

Results and Discussion. We were interested in finding further possibilities to trigger selectivity among the Siglecs Sialoadhesin, CD22 and MAG. Therefore we determined and compared the affinity of a small series of ligands using a surface plasmon resonance (SPR) based approach (Biacore).

Biacore assay. As reported previously, a Biacore assay for MAG[10] and CD22 (*see chapter 2.2.*) was performed by capturing the Siglec of interest on a protein A surface. For the reason of comparability, the assay set-up was maintained for Sialoadhesin. A pH scouting was performed in order to elucidate the appropriate pH for Sialoadhesin-capturing prior to protein A immobilization. After capturing and equilibration overnight around 4000 RU of Fc-Sn_{d1-3} were achieved. The reference cell provided only protein A to compensate for non-specific

binding to the matrix. The obtained sensorgrams were all negatives, as it was also observed for MAG, and therefore mirrored prior fitting.[10]

Compounds **1-3**[9,11] (Figure 1) were reported as ligands for Sialoadhesin, binding with 100-200 µM affinity. Furthermore, antagonists **1** and **2** were also tested for binding to CD22 and MAG, respectively.

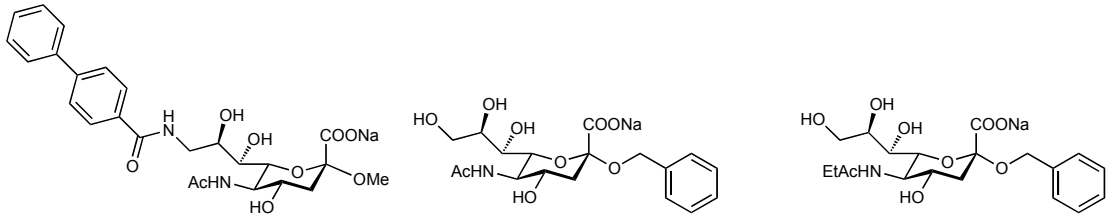


Figure 1. Overview on Sialoadhesin antagonists

Affinity determination. A small series of MAG and CD22 antagonists was evaluated. Firstly, the affinity of antagonist **4**, having the aglycon and the modification in 9-position of **1** and **2** combined, was determined. It bound with a K_D of 90 µM.

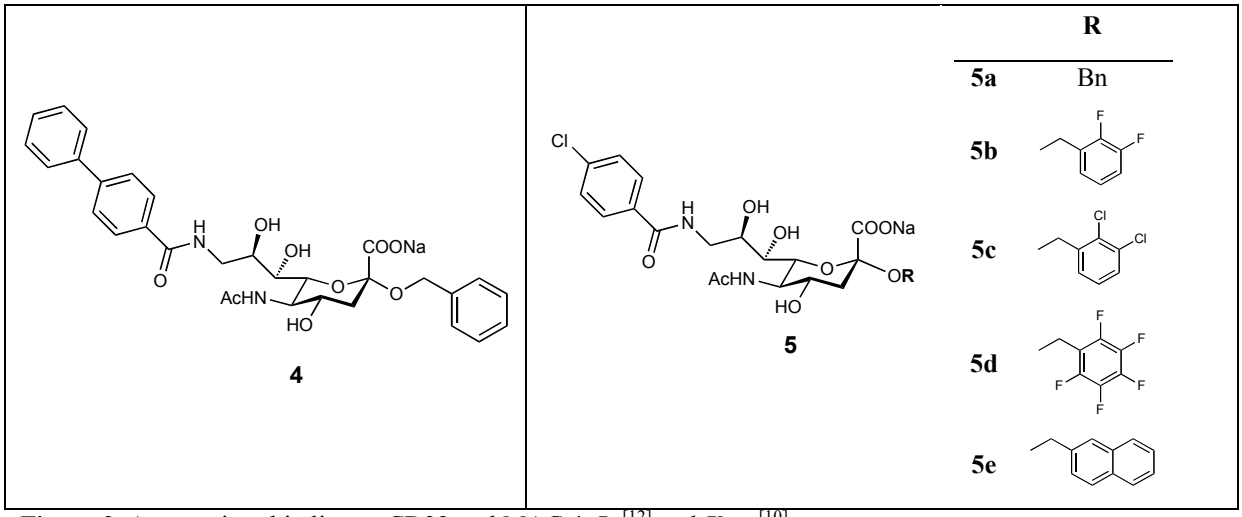


Figure 2. Antagonists, binding to CD22 and MAG **4**, **5a**[12] and **5b-e**.[10]

Afterwards, sialosides with the general formula **5** (Figure 2) were measured. Here, Sialoadhesin showed a clear preference for pentafluorobenzyl and 2,3-dichlorobenzyl as aglycon. Although **5c** and **5d** have 4-chlorobenzoyl instead of the preferred biphenylcarboxamide in 9-position, binding affinity was improved by a factor of 2.

Table 2. Affinities towards Sialoadhesin (Siglec-1), CD22 (Siglec-2) and MAG (Sigelc-4) were determined by Biacore (K_D) and hapten inhibition assay (rIC_{50})

	Sn-1	Sn-2		Sn-4	
Compound	**K_D [µM]**	**K_D [µM]**	**rIC_{50}**	**K_D [µM]**	**rIC_{50}**
1	--	--	0.034	--	--
4	90; 97	0.8	--	--	--
5a	--	--	1	25	1
5b	204	3.9	--	**2.4**	0.2
5c	56	**0.4**	--	4.3	0.3
5d	**49**	2.7	0.03	6.11	0.17
5e	71	2.8	--	11.6	0.5
6a	**63**	0.5	--	4.1	0.06
6b	--	1.4	--	--	0.09
6c	" 1000"[a]	1.2	0.03	17	0.4
6d	--	**0.1**	0.04	**1.4**	0.03

a. K_D was extrapolated without reaching saturation.

With respect to the 5-position Zaccai *et al.*[9] suggested based on X-ray and modeling data that small hydrophobic substituents in 5-position might increase binding affinity towards Sialoadhesin. This suggestion is in good agreement with our observations of a 4-fold improvement by replacing N*H*Ac with cyclopropylacetamide (→**6a**). In contrast, a mesyl-substitutent (**6c**) abolished binding almost completely.

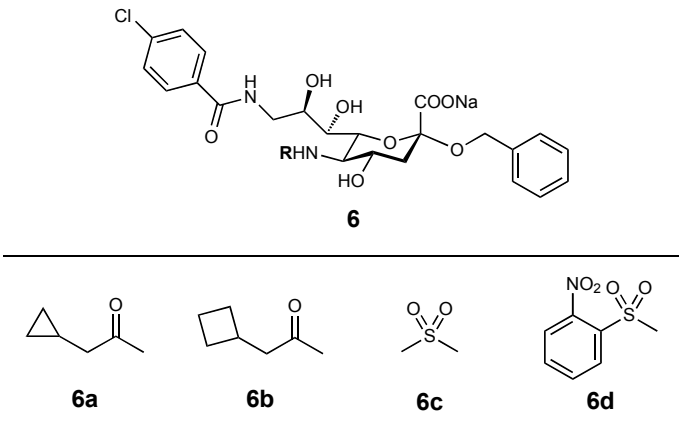


Figure 3. Ligands modified in 5-position.[10]

Antagonists **4**, **5c-e** and **6a** showed increased affinity towards Sialoadhesin, however binding is decreased by a factor of 10 -100 compared to CD22 and by a factor of 10 compared to MAG.

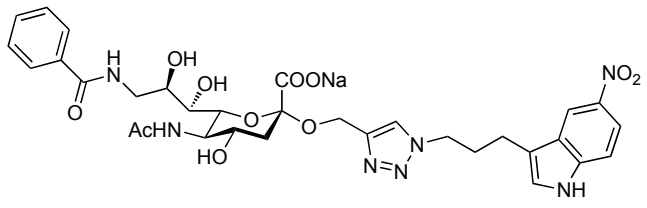
Achieving Selectivity - Trends.

9-position. Whereas biphenylcarboxamide in 9-position is tolerated by Sialoadhesin and CD22, affinity towards MAG drops significantly.[11,12] This might be due to a binding site, being spatially limited or due to a steric clash.[11] In Sialoadhesin, Zaccai *et al*. observed a rearrangement of Val109 upon binding of **1** compared to the unligated protein crystal structure.[11]

5-position. Sialoadhesin exhibits a preference for cyclopropylacetamide, improving binding by a factor of 3, whereas halogenated acetates were shown not to improve binding.[5] In the case of sialoside **6c**, having a mesyl-substituent in 5-position, binding affinity was almost lost. In contrast, sulfonamides were well tolerated by CD22 and MAG (**6c**,**d**, Table 1).[10] These findings are in good agreement with earlier findings, suggesting that the 5-position is suited for the achievement of selectivity. However, the differences between CD22 and MAG are not pronounced (*e.g.* antagonist **6d** binds with very similar affinity).

2-position. In all cases, the aglycon is clearly contributing to the binding affinity. However, the three Siglecs display similar preferences within the ligand-series and consequently, rather small substituents, such as benzyls, are not suited for the achievement of selectivity.

However, modifications in 2-position are readily introduced and a valuable approach was first presented by Shelke *at el*.[13] Based on a second site screening approach, selectivity was thought to rise from the different protein surfaces and shapes. When antagonist **7**, reported by Shelke *et al*.[13] was tested for binding to Sialoadhesin, no binding was observed.



7

Figure 4. Second site ligand designed for MAG displayed high selectivity

Conclusions.

Different approaches for the achievement of selectivity were discussed. Substituents in 5- but also in 9-position were observed to induce selectivity among Siglecs-1, -2 and -4. However, these effects depend on the substituent and not in all cases a clear preference for a specific Siglec was obtained. Therefore, the conduction of a second site screening for Sn and CD22 might a valuable approach for selective and high affinity ligands.

Experimental Part.

SPR analysis. The SPR measurements were performed on a Biacore 3000 surface plasmon resonance based optical biosensor (Biacore AB, Sweden). Sensor chips (CM5 and CM4), immobilization kits, maintenance supply and HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) were purchased from Biacore AB (HBS-EP ready-to-use; degassed and filtered). CM5 (CM4 respectively) chips were preconditioned prior to usage by injecting a series of conditioning solutions. A flow rate of 50 µl/min was used and 2 × 20 µl of 50 mM NaOH, 10 mM HCl, 0.1% SDS and 100 mM H_3PO_4 were injected. The carboxy groups on the CM5 (CM4) chip were activated for 10 min with a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.1 M 3-(*N*,*N*-dimethylamino)propyl-*N*-ethylcarbodiimide (EDC) at a flow rate of 10 µl/min. Protein A (P6031) was purchased from Sigma. A sample and a reference surface were prepared sequentially or in parallel. For immobilizing protein A, a stock solution (1 mg/ml in 50 mM phosphate buffer, pH 7.0) was diluted in 10 mM sodium acetate, pH 5.0 to obtain a concentration of 30 µg/ml. This solution was then injected over the activated surface for 10 min at a flow rate of 10 µl/min. Protein A densities around 4'000 RU and 5'000 RU were achieved. Flow cells were blocked with a 10 min injection of 1 M ethanolamine, pH 8.0.

For capturing, Sialoadhesin, CD22 and MAG_{d1-3}-Fc solution (expressed and purified as decribed)[14] were diluted to a 30-40 µg/ml concentration using NaOAc (pH 5). Afterwards, $Siglec_{d1-3}$-Fc (Siglec-1, -4) was injected at a flow rate of 1 µL/min for 10 min. $CD22_{d1-3}$-Fc was injected at a flow rate of 3 µL/min for 5 min. The surface was equilibrated over night at a flow rate of 5 µl/min, achieving densities around 2000 to 4000 RU.

Tenfold dilution series were freshly prepared in eluent buffer immediately before use. All binding experiments were conducted at 25 °C (except thermodynamic measurements) at a flow rate of 20 µl/min. The samples were injected over 1 min followed by 1 min dissociation. Each sample was measured with a duplicate of one concentration, using a randomized concentration order. Several buffer samples were injected before the first concentration, and

one blank between each concentration, which were used for the double blank referencing during data processing. Double referencing was applied to correct for bulk effects and other systematic artifacts. Data processing and equilibrium binding constant determinations were accomplished with Scrubber (BioLogic Software, Version 1.1g or 2.0a). Kinetic data were simultaneously fit using the non-linear regression program Clamp or Scrubber 2.0a.

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Second-site screening with CD22

A NMR-based second-site screening was performed and the ligands were evaluated with respect to their affinity.

Stefanie Mesch, Brian Cutting, Hendrik Koliwer-Brandl, Katrin Lemme, Oliver Schwardt, Soerge Kelm, Beat Ernst.

Work performed by Stefanie Mesch:
NMR-screening, synthesis of CD22 antagonists and performance and evaltuation of Biacore assay.

Second site screening CD22

Stefanie Mesch, Brian Cutting, Hendrik Koliwer-Brandl, Katrin Lemme, Oliver Schwardt, Soerge Kelm, Beat Ernst

Introduction.

CD22 is a member of the Siglec-family and is expressed on the surface of B-cells.[1] There, it regulates the activity of the B-cell receptor and is involved in regulation of B-cell survival[2] and homeostasis.[3] In most cases, CD22 is masked by *cis*-ligands, however *trans*-interactions are still possible.[4]

Multivalent synthetic sialosides have been reported to bind with enhanced affinity and therefore are able to overcome *cis*-interactions.[5] The approach of O'Reilly *et al.* showed that multivalent assembly of a bifunctional ligand could be triggered by an antibody.[6] Interestingly, they also showed that a dimer could overcome *cis*-interactions and therefore, we speculated that a sufficient potent monovalent antagonists may be also able to surmount *cis*-interactions. Furthermore, we wanted to develop an antagonist with two independent binding sites on CD22, what might be an interesting approach with respect for activity as well as for selectivity.

In recent years the concept of fragment assembly has gained increased importance in drug development. Here, fragments binding in close proximity are linked and the resulting hit is further refined. Consequently, various screening methods have been developed. A NMR-based approach[7] of second site screening has been successfully applied in the case of MAG.[8] Therefore we decided to apply this approach also to CD22.

Results and Discussion.

Structural interesting fragments were divided into sub-libraries, which were screened successively. Subsequent, verification of the identified hits was performed. The most promising fragment was then linked to the first site ligand, using different linker lengths resulting in antagonists **16a-h**.

The screening approach is based on differences in the relaxation rates of T1rho of the free and bound fragment. As the longitudinal relaxation (T1rho) of molecules depends on their size, after addition of protein, the fragments binding to the protein adopt the slower relaxation. However, for the identification of only those fragments, which are binding close

to the binding site, first-site ligand **6** with a TEMPO-substituent in 2-position was added. Due to the unpaired electron, t1rho relaxation rates of compounds binding in close proximity (ca 10 Å) are changed. Upon addition of *L*-ascorbic acid the tempo is reduced and relaxation returns to "normal". This procedure was applied to all sub-libraries.

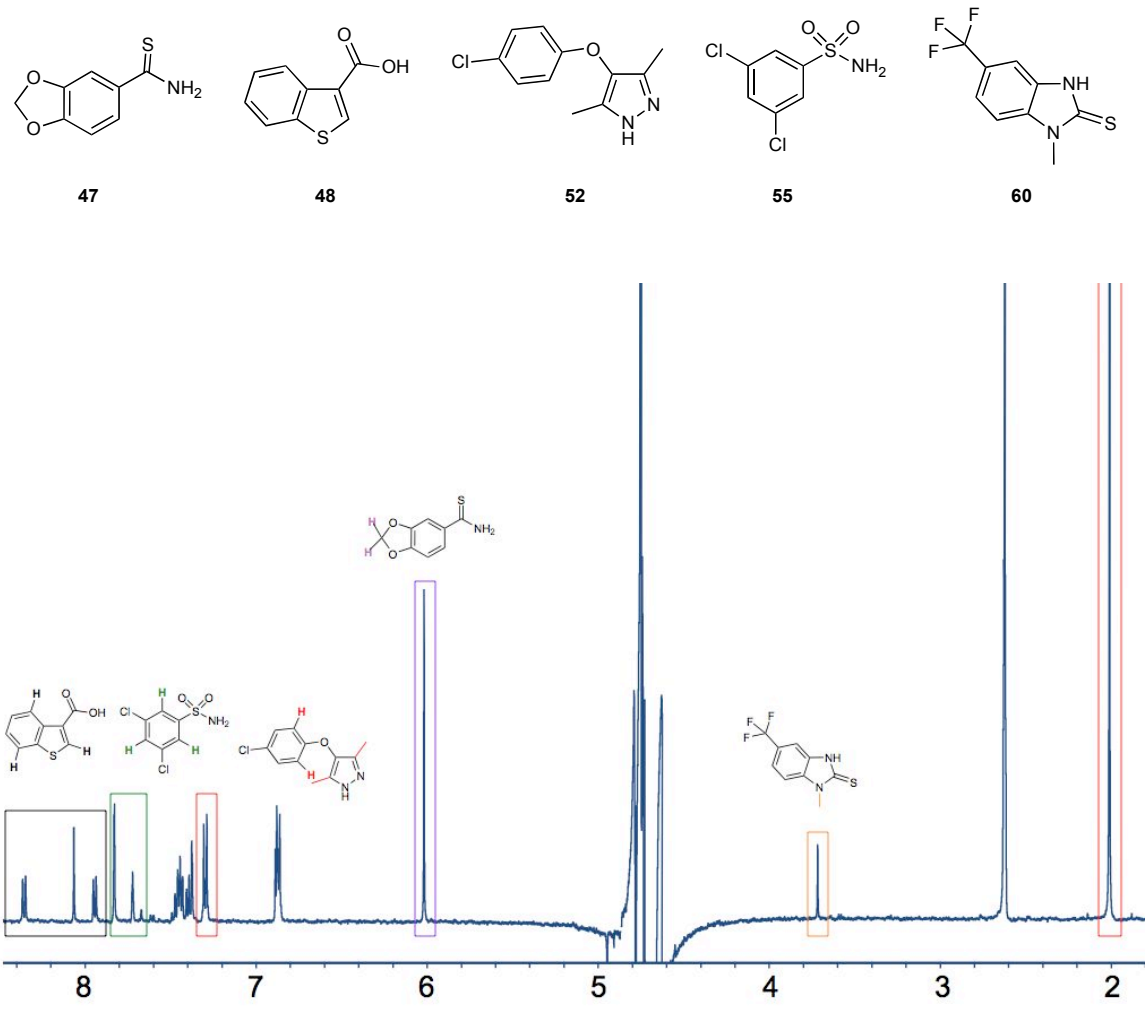
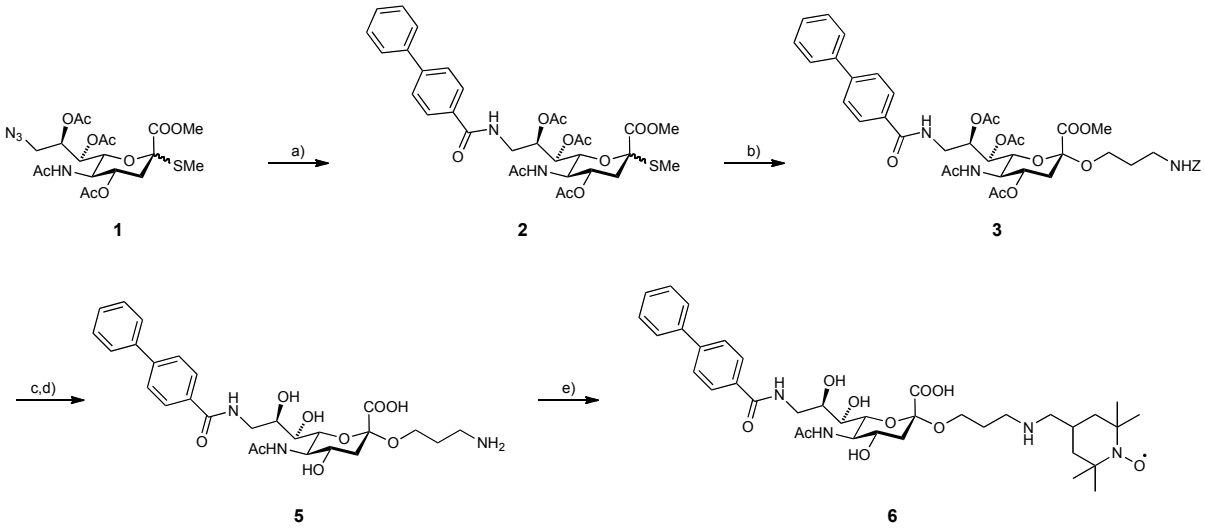


Figure 1. Exemplary sub-library and the corresponding ^{1}H-spectra.

Synthesis of TEMPO-ligand.

For our attempt to identify small molecules binding close to the first site ligand, TEMPO (free radical) was attached to the first site ligand at the reducing end (Scheme 1).



Scheme 1. a) 4-Biphenylcarbonyl chloride, PPh_3, DCE, rt (77%); b) 3-Z-amino-propan-1-ol, NIS, TfOH, MeCN (39%); c) 10% aq. NaOH, MeOH; d) $Pd(OH)_2/C$, H_2, MeOH, 1 bar, rt, 2 h (73% two steps); e) 4-Carboxy-TEMPO (free radical), HBTU, HOBt, DMF, rt, 2 h (28%). **1** was prepared according to Mesch *et al*., **2010**.[9]

NMR screening.

In a first step we verified that **6** is binding to CD22. Hence, selective T1 inversion recovery was measured for the reduced form of **6** in absence and presence of the protein. As the relaxation behavior differs for big and small molecules, binding of the ligand to the protein induced an acceleration of the relaxation rate. We observed a distinct different relaxation behavior upon addition of CD22 to the ligand (Figure 2), indicating binding.

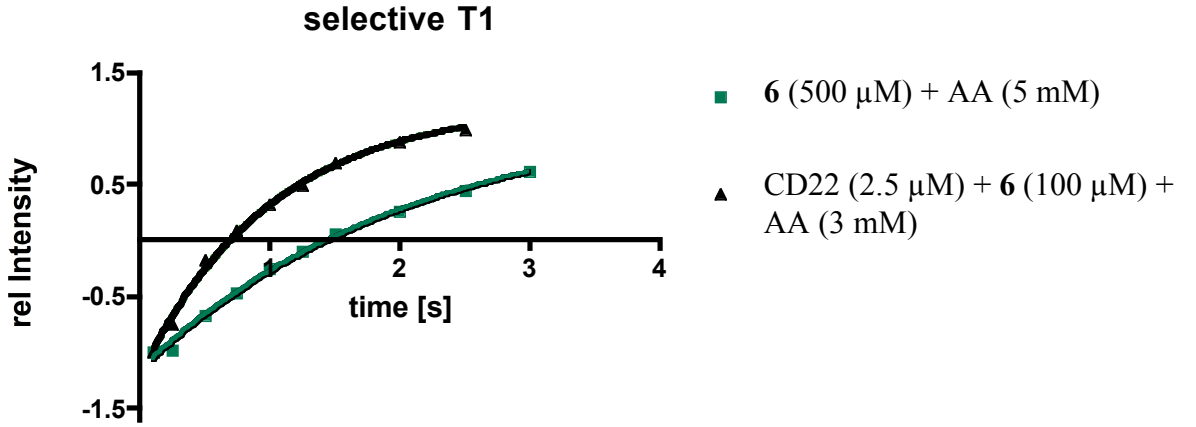


Figure 2. Selective T1 inversion recovery measurement shows binding of **6** (reduced form); AA: *L*-ascorbic acid.

Afterwards, T1rho of all components of the different sub-libraries were measured in the absence and then in the presence of the TEMPO first-site ligand (**6**). As a control, *L*-ascorbic

acid is added and the T1rho of the fragments in the presence of the reduced form of the TEMPO-ligand is measured.

The experiments were conducted using 300 µM of the second site fragment, 100 µM SMC-9-8 (TEMPO-ligand) and 2.5 µM CD22 (80.1 µl of 1mg/ml protein solution in PBS) in PBS/D_2O (1/1, V_t 250 ul). For the reduction *L*-ascorbic acid sodium salt (6 mM) was added. In order to get a reasonable signal to noise ratio, 256 scans per experiment were applied.

Finally, 4 compounds were identified as possible hits (Figure 3) and were subjected to further investigations for the purpose of verification. Furthermore, a qualitative ranking between the compounds was established based on the "in-proximity of the tempo binding effect".

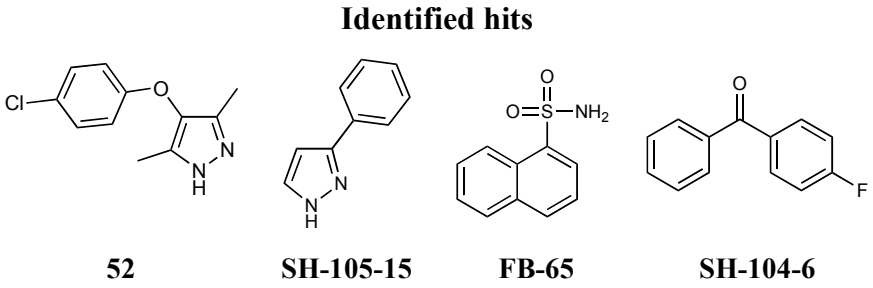


Figure 3. Fragments were identified as preliminary hits after screening the library

The transverse relaxation (T1rho) of fragment **52** was re-measured at 10 time points. Although the decay was faster in the presence of the active TEMPO-compound and consequently bound in close proximity to the first binding site, the decay was slightly slower than with the protein alone.

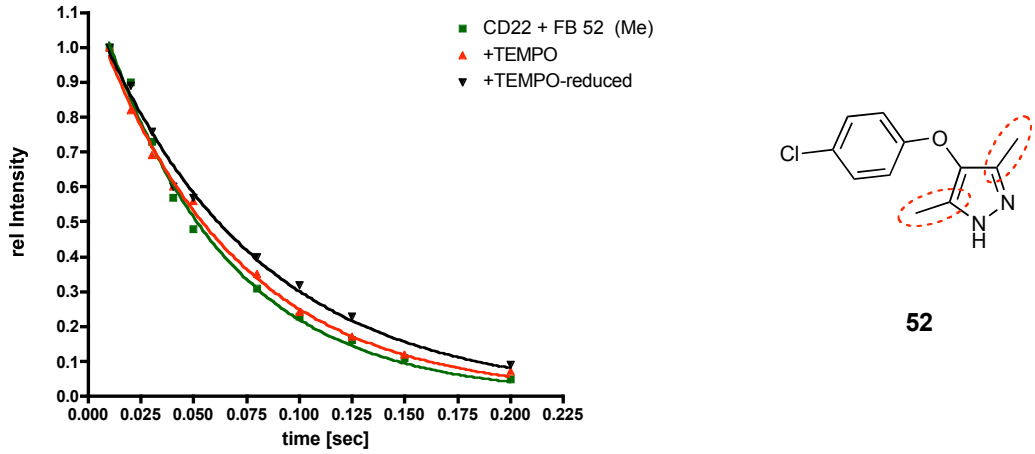


Figure 4. Decay of transverse magnetization (T1rho); For evaluation the methyl groups were chosen.

Based on this observation, we suspected that **52** is competing to a certain extend with the TEMPO-ligand for the binding site. Therefore, we measured the decay of T1rho of **52** in the presence of CD22 as a control and added afterwards **6** (Figure 5). Upon addition of the reduced TEMPO-ligand, again, we observed that the relaxation rate of **52** was slowed down. These results show that **52**, being a weaker binder than **6**, is replaced. However, there seems to be a second binding site for **52**, as there is a visible difference between the rate of the free ligand and after addition of the competitor (this would not be the case if it was only binding in the "first-binding site").

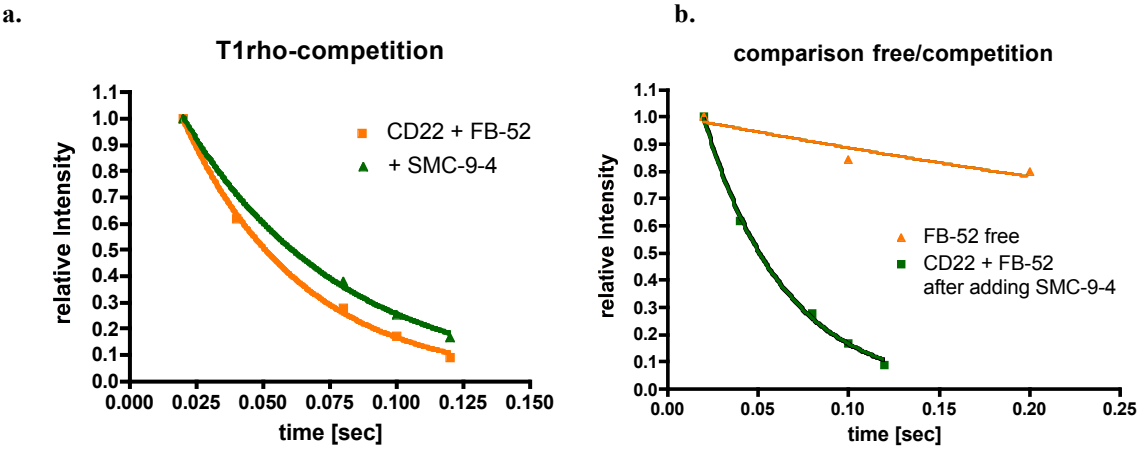


Figure 5. a. Influence on the T1rho by addition of SMC-9-4. **b.** decay of free A and A with CD22 and SMC-9-4.

Beside **52**, naphtalene-1-sulfonamide (**FB-65**) and 4-fluorobenzophenone (**SH-104-6**) have been identified as possible hits and were therefore re-measured one by one.

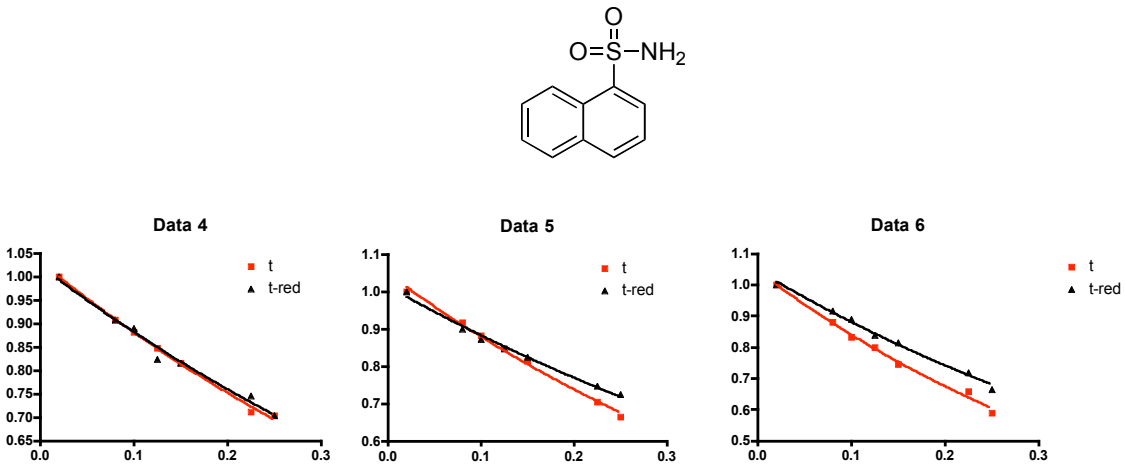


Figure 6. Decay of T1rho in the presence of **6** (red) and **6**-reduced (black) at 10, 20, 30, 40, 50, 80, 100 µs.

The measurement showed an influence of compound **6** on the relaxation of **FB 65** although only one of three (analyzed) protons shows a distinct difference. In the case of 4-Fluorobenzophenone (**SH-104-6**) all three protons investigated showed a clear influence (Figure 7). We considered **SH-104-6**, that showed the most promising "in-proximity of the tempo binding effect", to be further investigated.

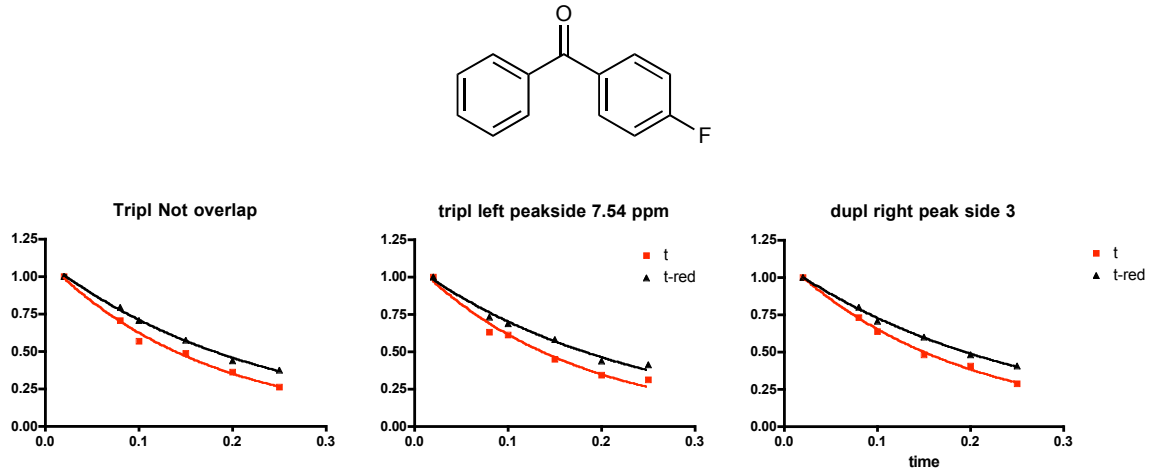


Figure 7. Decay of T1rho in the presence of **6** (red) and **6**-reduced (black) at 10, 20, 30, 40, 50, 80, 100 µs.

We were interested in the orientation of the second site fragment with respect to the TEMPO-ligand. In order to figure the directionality out, equation (1) was used. Here, high values of x indicate a closer proximity towards the free radical.

$$x = \frac{T1rho^{red} - T1rho^{rad}}{T1rho^{red}} \qquad (1)$$

When this concept was applied to **SH-104-6** it turned out that the fluorine points towards the TEMPO.

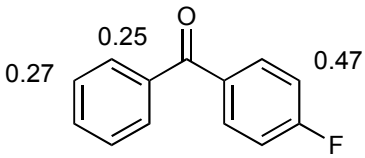


Figure 8. Fluorine seems to point towards the TEMPO-ligand as the influence on neighboured proton is distinct higher compared to the others.

With respect to facile chemical linking, 4-hydroxybenzophenone, 3-hydroxybenzophenone and 2-hydroxy-9-fuorenone were investigated. Unfortunately, the latter compound was completely insoluble in the buffer and therefore not further considered.

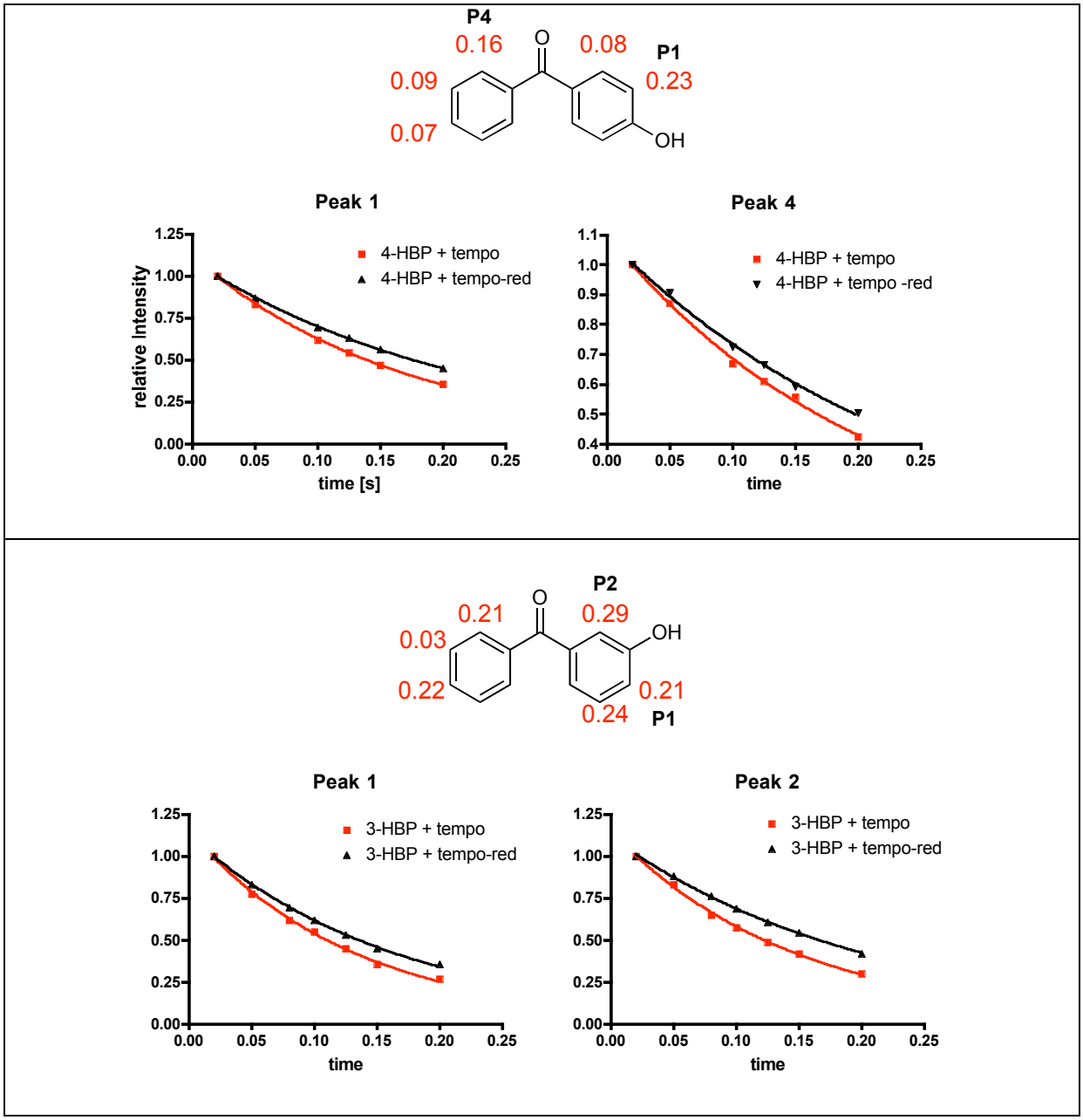
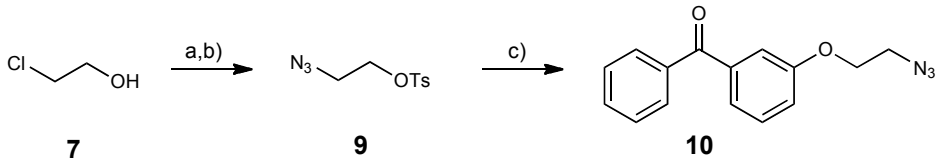


Figure 9. Decay of T1rho in the presence of **6** (red) and **6**-reduced (black) at 10, 20, 30, 40, 50, 80, 100 µs. Evaluation of several protons hints on the orientation of the fragment with respect to the TEMPO-ligand.

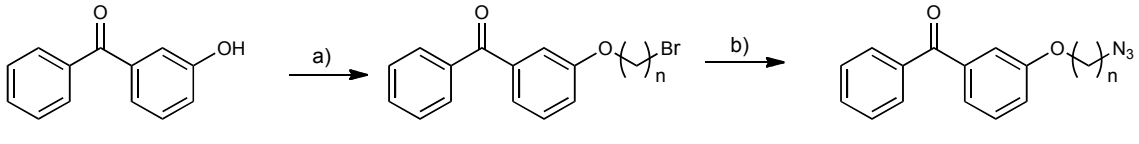
4-hydroxybenzophenone as well as 3-hydroxybenzophenone showed a clear effect of the TEMPO-radical on the T1rho rates. Finally, 3-Hydroxybenzophenone was chosen due to good binding and synthetic reasons.

Synthesis of second site fragments was performed by alkylating 3-Hydroxybenzophenone with the corresponding bromides (**12a**,**b**, Scheme 3). Due to stability reasons of 1,2-dibromoethane, the 2C-linker was obtained starting from commercially available 2-chloroethanol by nucleophilic substitution of the chloride by azide[10] followed by tosylation.[11]



7 **9** **10**

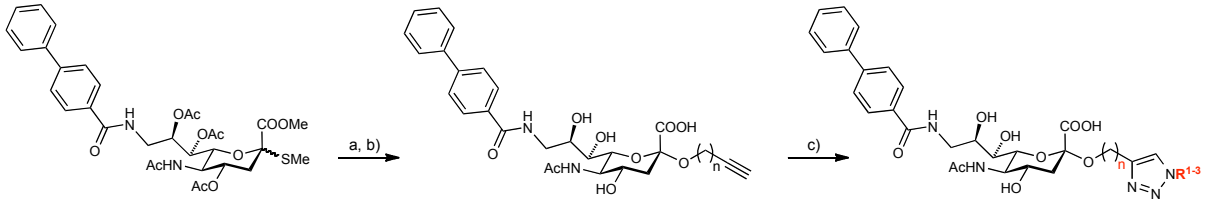
Scheme 2. a) NaN_3, H_2O (quant);[10] b) *p*TsCl, DCM (91%);[11] c) 3-Hydroxybenzophenone (**11**), NaH, DMF (85%).



11 **12a**: n = 3 **12b**: n = 4 **13a**: n = 3 **13b**: n = 4

Scheme 3. a) 1,4-Dibromobutane (→**12a**) or 1,5-Dibromopentane (→**12b**), K_2CO_3, MeOH (**12a**: 74%, **12b**: 78%); b) NaN_3, DMF (**13a**: 70%, **13b**: 95%).

Synthesis of first-site ligands. Antagonists **15a-c** with alkyne linkers (1C to 3C) were synthesized by glycosidation of donor **2** with the corresponding alcohols using the promotor system NIS and triflic acid.



2 **15a**: n = 1 **15b**: n = 2 **15c**: n = 3 **16a-h**

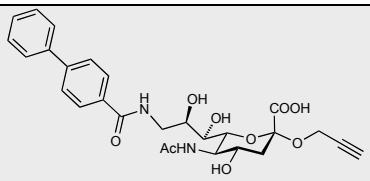
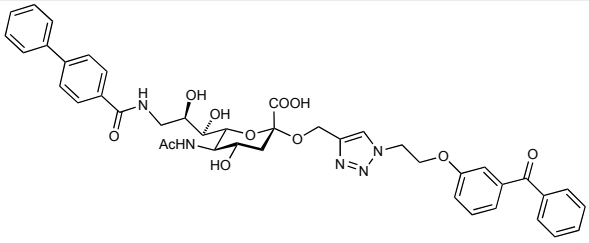
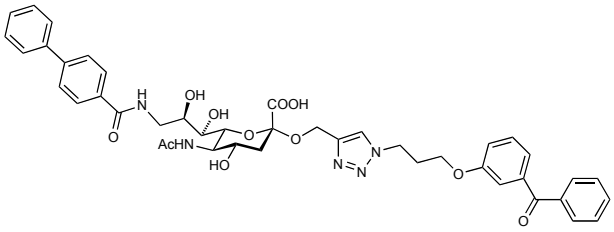
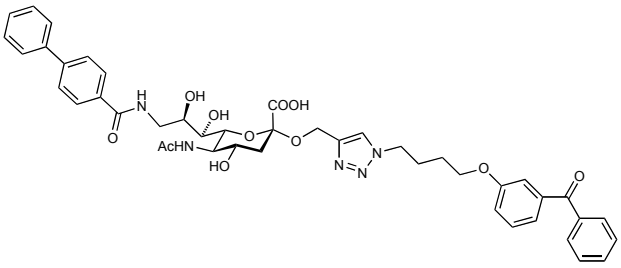
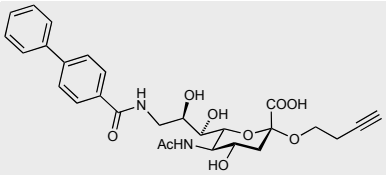
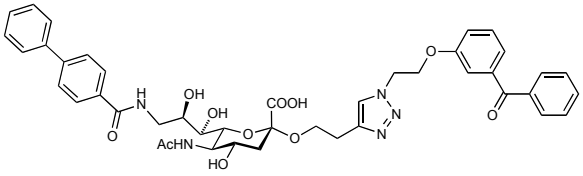
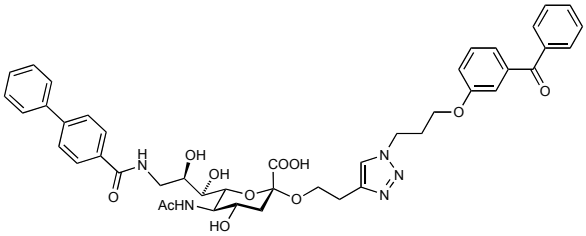
Scheme 4. a) alcohol, NIS, TfOH, MeCN (45-73%); b) 10% aq. NaOH, MeOH (50-80%); c) **10** (or i. **13a**; or ii. **13b**), $CuSO_4 \cdot 5H_2O$, *L*-ascorbic acid sodium salt, ^{t}BuOH/ H_2O/ THF (1/1/1), rt, (12-54%); n=1,2,3.

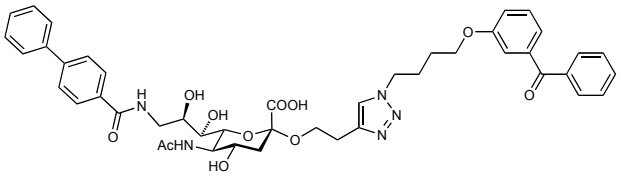
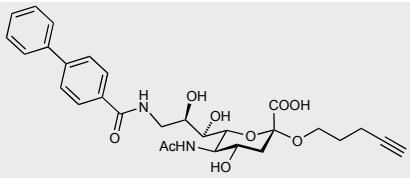
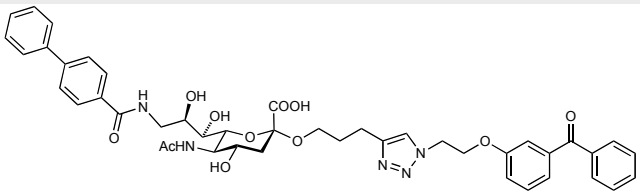
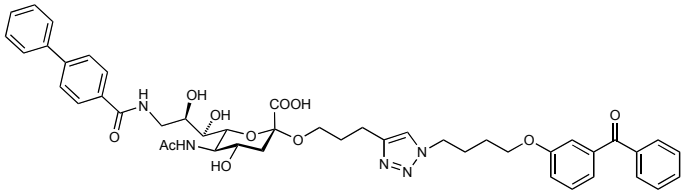
Afterwards, the sialosides were reacted under Cu(I)- catalyzed click-conditions with the azides **15a-c** to afford the *anti*-configurated final compounds **16a-h**.

Surface plasmon resonance (SPR). The final compounds **16a-h** as well as sialosides **15a-c** were evaluated by a surface plasmon resonance based assay (Biacore). Here, Fc-$CD22_{d1-3}$ was captured on a protein A surface and the ligands were injected over the surface.

Unfortunately, the binding affinity of the second-site compounds compared to the first site ligands with the acetylene linker was decreased by a factor of 3 to 8. Moreover, some compounds abolished completely binding (**16b**,**c** and **16h**, Table 1).

Table 1. Overview on second site library, K_D determined using CD22@31.10.09

Compound	Structure	n	K_D [µM]
15a		1	2.3
16a		1	"28"
16b		1	n.b.[a]
16c		1	n.b.[a]
15b		2	0.33
16d		2	3.1
16e		2	2.6

16f		2	4.1
15c		3	0.46
16g		3	1.4
16h		3	n.b.[a]

BP: biphenyl; [a]n.b. not binding.

These results were suspicious, as the protein batch used in the screening was not the same as used for the Biacore assay. Consequently, we hypothesized, that the screening was performed using a partially unfolded protein, leading to a false positive hit identification. This was confirmed by MS measurements of the two proteins. Batch 1 (NMR) of CD22 could hardly be ionized, indicating that is was unfolded, whereas in contrast, CD22-batch 2 (Biacore) was readily ionized and yielded the expected molecular weight.

As a consequence, we repeated the screening, using the intact protein. Unfortunately, repetition of the T1rho experiments using the "identified" hit 3-Hydroxybenzophenone revealed, that it was not binding to the CD22 at all, as no difference in the relaxation rates after 20 and 200 msec was observed.

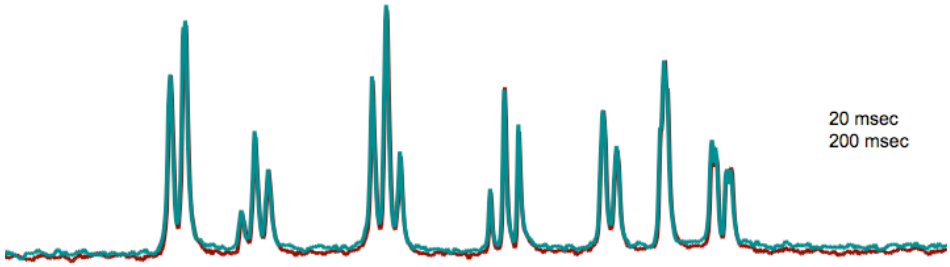


Figure 10. Overlay of T1rho spectra with a delay of 20 and 200 msec; NMR sample: **3-HBP** (300 µM), CD22 (2.5 µM).

In a next step, we repeated T1rho experiments using a mix screened before, containing hits identified earlier. Also here, only a marginal difference in the relaxation after 20 and 200 msec was observed and consequently the fragments were binding to the protein in a negligible extend.

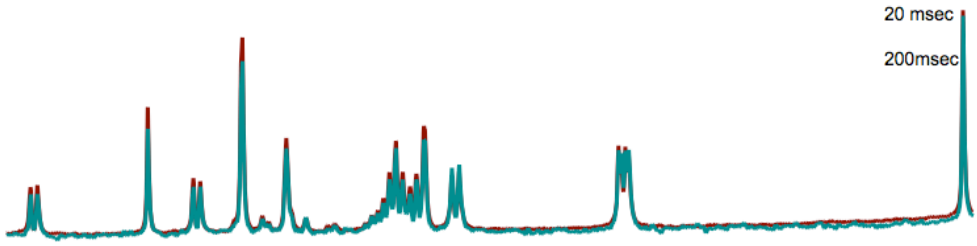


Figure 11. Overlay of T1rho spectra of mix 4; NMR sample: Mix 4 (300 µM each compound) + CD22 (2.5 µM).

Finally, selective T1 experiments, using the reduced TEMPO-ligand, were repeated. The selective T1 inversion recovery did not show a distinct discrimination of the relaxation behaviour in absence or presence of the protein.

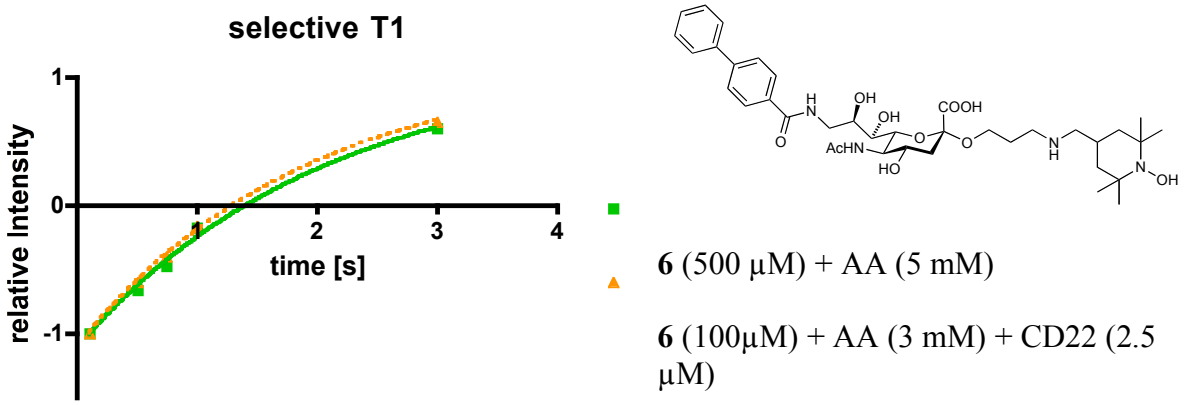
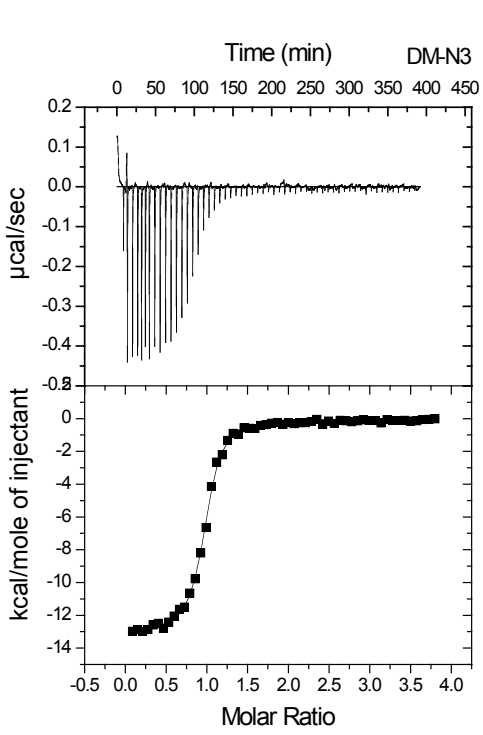
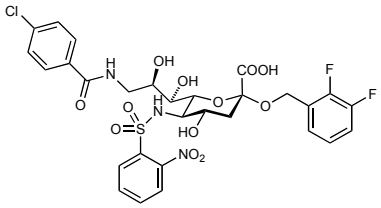


Figure 12. Selective T1 inversion recovery of TEMPO-ligand

Hereupon, we verified by MS that the ligand **6** in the NMR-sample was not decomposed and by SDS page that the protein in NMR tube was not degraded.

Afterwards, STD NMR was performed with CD22 (2.5 µM) and **6** (1.5 mM). No signal was obtained, suggesting that the ligand is not binding.
However, binding of ligand **6** was determined in the Biacore assay to be 800 nM. In order to clarify these conflicting results, we additionally performed an ITC measurement of antagonist **23**. As the ITC and Biacore assay gave similar K_D values, we speculated that the ligand bound too tightly to the protein and therefore the NMR approach was not suited for this "affinity" range.





N	ΔG [kJ/mol]	ΔH [kJ/mol]	TΔS [kJ/mol]	K_D [nM]	K_D [nM]
0.96	-39.3	-54.8	-15.5	131	105

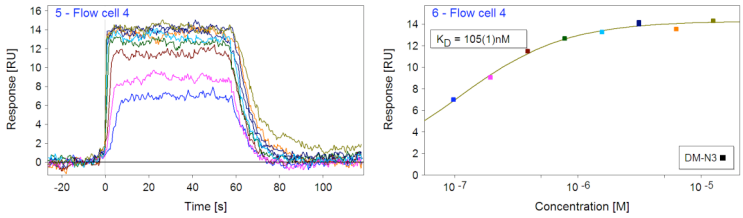


Figure 13. ITC measurements confirm the results obtained by SPR-assay.

Conclusion.

A second-site screening was performed and antagonists were subsequently synthesized and evaluated. As the binding affinity was decreased, in contrast to our expectations, further investigations revealed that the used CD22 was unfolded (at least partially). All NMR experiments were repeated, however we could not identify new hits an even the first site ligand could not be investigated with the above described approaches.

Biacore and ITC measurements revealed a low μM affinity for the TEMPO-ligand and therefore we concluded that the "negative" results in the NMR assay might be explained by the high affinity of the ligand, being beyond the range suited for this method.

Experimental Part.

Chemistry. NMR spectra were recorded on a Bruker Avance DMX-500 (500 MHz) spectrometer. Assignment of ^{1}H and ^{13}C NMR spectra was achieved using 2D methods (COSY, HSQC, TOCSY, ROESY, NOESY). Chemical shifts are expressed in ppm using residual $CHCl_3$, CHD_2OD, CHD_2CN and HDO as references. Optical rotations were measured using Perkin-Elmer Polarimeters 241 and 341. MS analyses were carried out using a Waters Micromass ZQ Detector system. The spectra were recorded in positive or negative ESI mode. Reactions were monitored by TLC using glass plates coated with silica gel 60 F_{254} (Merck) and visualized by using UV light and/or by charring with a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aq 10% H_2SO_4). Column chromatography was performed on silica gel (Fluka, 40-60 mesh). Methanol was dried by refluxing with sodium methoxide and distilled immediately before use. Pyridine was freshly distilled under argon over CaH_2. Dichloromethane (DCM), dichloroethane (DCE), acetonitrile (MeCN), toluene, and benzene were dried by filtration over Al_2O_3 (Fluka, type 5016 A basic). Molecular sieves (3 or 4 Å) were activated in vacuo at 500 °C for 2 h immediately before use. Synthesis of compound **1** was done according to a published procedure.[12] Compound **2** was obtained as described in *Chapter 2.2*. 2-Azidoethanol[10] and subsequently 1-[(2-azidoethoxy)sulfonyl]-4-methylbenzene[11] were prepared according to published procedures. Compound **11** was purchased from Sigma-Aldrich.

Methyl [3-benzoxycarbonylaminopropyl 5-acetamido-4,7,8-tri-*O*-acetyl -3,5,9-trideoxy -9- (4-phenyl)benzamido) -D -glycero-α-D -galacto-2-nonulopyranosid]onate (3). Compound **2** (150 mg, 0.23 mmol, 1.0 eq) was reacted with benzyl *N*-(3-hydroxypropyl)carbamate (145 mg, 0.68 mmol, 3.0 eq), NIS (82 mg, 0.37 mmol, 1.6 eq) and triflic acid (16 μL, 0.18 mmol, 0.8 eq) according to general procedure A to yield **3** after chromatography on silica gel (5% gradient of MeOH in DCM) as white solid (72 mg, 38%). $[\alpha]^{20}_{D}$ -2.4 (*c* 0.35, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.69 (m, 2H, CH_2), 1.78 (s, 3H, NHAc), 1.87 (t, *J* = 12.6 Hz, 1H, H-3a), 1.91, 1.93, 2.16 (3 s, 9H, 3 OAc), 2.40 (dd, *J* = 4.9,

12.8 Hz, 1H, H-3b), 3.01 (td, *J* = 4.0, 11.0 Hz, 1H, H-9a), 3.24 – 3.37 (m, 2H, CH_2), 3.39 – 3.53 (m, 2H, CH_2), 3.69 (s, 3H, OMe), 3.92 (d, *J* = 10.5, 1H, H-6), 4.12 (m, 1H, H-5), 4.43 (m, 1H, H-9b), 4.91 – 5.11 (m, 2H, OC*H*$_2$Ar), 5.11 – 5.30 (m, 3H, H-4, H-7, H-8), 6.10 (d, *J* = 10.0 Hz, 1H, N*H*Ac), 7.12 (d, *J* = 5.9 Hz, 3H, 9-NH, CH_{ar}), 7.25 (m, 3H, CH_{ar}), 7.30 (t, *J* = 7.3 Hz, 1H, CH_{ar}), 7.37 (t, *J* = 7.5 Hz, 2H, CH_{ar}), 7.44 – 7.54 (m, 4H, CH_{ar}), 7.79 (AA'BB' of AA'BB', *J* = 8.2 Hz, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 20.9, 21.0, 21.1 (3 OAc), 23.1 (NHAc), 29.3 (CH_2), 38.0 (C-3), 38.3 (C-9), 48.9 (C-5), 52.8 (OMe), 60.4 (CH_2), 62.3 (CH_2), 66.6 (O*C*H$_2$Ar), 68.3, 68.7 (C-7, C-8), 69.0 (C-4), 71.0 (C-6), 98.5 (C-2), 127.2, 127.3, 127.5, 127.8, 128.0, 128.5, 128.6, 128.9, 132.8, 136.3, 136.6, 139.9, 144.4 (18C, C-Ar), 156.9 (CONH), 167.3, 167.8, 168.3, 170.4, 170.8, 171.9 (6 CO). HRMS calcd. for $C_{42}H_{49}N_3O_{14}$ $[M+Na]^+$: 842.3112; found m/z 842.3114.

Sodium [3-benzoxycarbonylaminopropyl 5-acetamido -3,5,9-trideoxy-9-(4-phenyl) benzamido)-D -glycero-α-D -galacto-2-nonulopyranosid]onate (4). Compound **3** (72 mg, 89 μmol, 1.0 eq) in MeOH (3 mL) was treated with 10% aq. NaOH (0.2 mL) as described in procedure B to yield **4**, which was directly reacted further. $[\alpha]^{20}_D$ -12.8 (*c* 0.5, MeOH). ^{1}H NMR (500 MHz, D_2O) δ 1.62 (m, 3H, H-3a, CH_2), 1.96 (s, 3H, NHAc), 2.66 (dd, *J* = 4.7, 12.3 Hz, 1H, H-3b), 3.07 (m, 2H, CH_2), 3.41 (m, 2H, CH_2), 3.50 (d, *J* = 8.9 Hz, 1H, H-7), 3.62 (m, 1H, H-4), 3.65 – 3.83 (m, 4H, H-5, H-6, H-9a, H-9b), 3.98 (m, 1H, H-8), 7.11 (d, *J* = 7.2 Hz, 2H, CH_{ar}), 7.19 (m, 3H, CH_{ar}), 7.39 (t, *J* = 7.3 Hz, 1H, CH_{ar}), 7.45 (t, *J* = 7.5 Hz, 2H, CH_{ar}), 7.58 (d, *J* = 7.8 Hz, 4H, CH_{ar}), 7.69 (d, *J* = 8.3 Hz, 2H, CH_{ar}). ^{13}C NMR (D_2O) δ 22.0 (NHAc), 37.3 (CH_2), 40.4 (C-3), 43.0 (C-9), 51.9 (C-5), 62.1 (CH_2), 66.5 (CH_2), 68.3, 70.2, 71.0 (C-4, C-7, C-8), 72.5 (C-6), 92.0 (C-2), 127.0, 127.1, 127.7, 128.0, 128.3, 128.6, 129.1, 139.4 (18C, C-Ar), 150.0 (CONH), 175.0, 175.3 (3C, CO). HRMS calcd. for $C_{35}H_{40}N_3NaO_{11}$ $[M+Na]^+$: 724.2451; found m/z 724.2458.

Sodium [3-aminopropyl 5-acetamido -3,5,9-trideoxy-9-(4-phenyl)benzamido-D -glycero-α-D -galacto-2-nonulopyranosid]onate (5). Compound **4** was dissolved in dry MeOH (4 mL) and $Pd(OH)_2$/C (Degussa, 40 mg) was added under argon. Afterwards the mixture was stirred under H_2 (1bar) for 2 h. After completion of the reaction, the catalyst was removed by filtration over a pad of celite and the pure product was obtained after chromatography on RP-18 (5% gradient of MeOH in water) to yield **5** (35 mg, 73% 2 steps). $[\alpha]^{20}_D$ -13.1 (*c* 0.33, MeOH). ^{1}H NMR (500 MHz, CD_3OD/H_2O) δ 1.74 (m, 1H, H-3a), 1.92 (m, 2H, CH_2), 2.00

(m, 3H, NHAc), 2.34 (dd, *J* = 4.7, 12.9 Hz, 1H, H-3b), 3.06 (m, 2H, CH_2), 3.51 (d, *J* = 9.2 Hz, 1H, H-7), 3.54 – 3.69 (m, 1H, H-9a), 3.84 – 3.97 (m, 5H, H-5, H-6, H-9b, OCH_2), 4.08 (m, 2H, H-4, H-8), 7.40 (m, 2H, CH_{ar}), 7.47 (m, 2H, CH_{ar}), 7.65 (m, 2H, CH_{ar}), 7.72 (m, 2H, CH_{ar}), 7.96 (m, 2H, CH_{ar}). ^{13}C NMR (CD_3OD/H_2O) δ 23.0 (NHAc), 27.6 (CH_2), 40.0 (CH_2), 42.2 (C-3), 44.2 (C-9), 53.6 (C-5), 63.1 (OCH_2), 67.9 (C-4), 70.4 (C-8), 71.8 (C-7), 74.1 (C-6), 101.9 (C-2), 128.0, 129.1, 130.0, 133.7, 140.9, 145.5 (12C, C-Ar), 174.7, 176.5, 180.9 (3 CO). ESI-MS calcd. for $C_{27}H_{35}N_3O_9$ $[M+Na]^+$: 568.57; found m/z 568.30.

Sodium [3-(2,2,6,6-tetramethylpiperidine-1-oxyl-4-carboxamido)propyl 5-acetamido -3,5,9-trideoxy-9-(4-phenyl)benzamido)-D-glycero-α-D-galacto-2-nonulopyranosid]onate (6). Compound **5** (25 mg, 45 µmol, 1.0 eq) was added to the reaction mixture of 4-Carboxy-2,2,6,6-tetramethylpiperidine 1-oxyl (15 mg, 73 µmol, 1.6 eq), HOBt (11 mg, 81 µmol, 1.8 eq), HBTU (17 mg, 45 µmol, 1.0 eq) and DIPEA (0.3 mL) in dry DMF (1 mL). After 2 h, the solvent was removed under high vacuum and the pure product was obtained after LC-MS purification as reddish solid (9 mg, 28%).

General procedure of glycosidation (A).

Compound **2** (0.33 mmol) was dissolved in dry acetonitrile (2.0 ml) under argon. The alcohol (0.99 mmol) and powdered MS 3Å were added. The mixture was stirred at r.t. for 1.5 h. Then the suspension was cooled to -40 °C and subsequently treated with *N*-iodosuccinimide (0.53 mmol) and triflic acid (0.26 mmol). After 30 min the reaction mixture was warmed to -30 °C and stirring continued for 24 h. The mixture was then warmed to r.t., stirred for another 2 h and filtered through a pad of celite. The celite was washed with DCM (10 ml) and the filtrate was subsequently washed with 20% aq. $Na_2S_2O_3$ (1 ml) and saturated aqueous $NaHCO_3$ (3 × 5 ml). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel.

General procedure of deprotection (B).

Compound **3** (**15**, **17**, **19**) (0.13 mmol) was dissolved in MeOH (5.0 ml) and treated with 10% aq. NaOH (0.4 ml). The reaction mixture was stirred at r.t. for 3 h. Then the reaction mixture was neutralized with 7% aq. HCl. The solvent was evaporated and the crude product was purified LCMS.

General procedure Cu(I)-catalyzed click-reaction (C).

To a mixture of acetylene (13 µmol) in degassed ^{t}BuOH:H_2O (v/v, 1:1), the corresponding azide (21 µmol), cupric sulfate pentahydrate (3.3 µmol) and sodium ascorbate (7.5 µmol) were added under argon atmosphere. The reaction mixture was stirred at r.t. overnight. Afterwards, the solvent was removed under reduced pressure and the residue was purified LCMS.

[3-(2-azidoethoxy)phenyl](phenyl)methanone (10). 3-Hydroxybenzophenone (**11**, 150 mg, 0.76 mmol, 1.0 eq) was dissolved in DMF (2 mL). After cooling to 0 °C, NaH (52 mg, 1.52 mmol, 2.0 eq) was added and stirring was continued for 30 min. 1-[(2-azidoethoxy)sulfonyl]-4-methylbenzene (274 mg, 1.14 mmol, 1.5 eq) in DMF (1mL) was added dropwise and the reaction was allowed to come to r.t. After 24 h, ethyl acetate 4 mL) was added and the organic layer was washed with brine (2 × 1 mL) and water (1 × 1 mL), dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The pure product was obtained after chromatography on silica gel (petrol ether/ ethyl acetate 3:1) as colorless oil (172 mg, 85%). IR (NaCl) ν 2109 (N_3), 1658 (CO) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.57 – 3.65 (m, 2H, CH_2N_3), 4.17 – 4.22 (m, 2H, OCH_2), 7.14 – 7.19 (m, 1H, CH_{ar}), 7.34 – 7.43 (m, 3H, CH_{ar}), 7.47 – 7.50 (m, 2H, CH_{ar}), 7.55 – 7.62 (m, 1H, CH_{ar}), 7.80 – 7.81 (m, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 50.1 (CH_2N_3), 67.2 (OCH_2), 114.8, 119.6, 123.6, 128.3, 129.4, 130.0, 132.5, 137.5, 139.0, 158.3 (12C, C-Ar), 196.3 (CO). ESI-MS calcd. for $C_{15}H_{13}N_3O_2$ $[M+Na]^+$: 290.08; found m/z 290.05. Analysis calcd. For $C_{15}H_{13}N_3O_2 \cdot ¼\ H_2O$, C 66.29, H 5.01, N 15.46; found C 66.40, H 5.25, N 14.97.

(3-(3-bromopropoxy)phenyl)(phenyl)methanone (12a). 3-Hydroxybenzophenone (**11**, 100 mg, 0.5 mmol, 1.0 eq) was dissolved in MeOH (2 ml) and 1,3-dibromopropane (253 µl, 503 mg, 2.5 mmol, 5.0 eq) and potassium carbonate (138 mg, 1.0 mmol, 2.0 eq) were added successively. The reaction mixture was stirred at 50 °C for 5 h. After cooling to r.t., the solvent was evaporated and ethyl acetate was added. Then, the organic layer was washed with brine and water, dried over Na_2SO_4, filtered and the solvents were removed under reduced pressure. The crude product was purified by chromatography on silica gel (petrol ether/ ethyl acetate 4/1) to afford **12a** (119 mg, 74%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.32 (p, J = 6.0 Hz, 2H, CH_2), 3.60 (t, J = 6.4 Hz, 2H, CH_2Br), 4.15 (t, J = 5.8 Hz, 2H, OCH_2), 7.04 – 7.20 (m, 1H, CH_{ar}), 7.31 – 7.41 (m, 3H, CH_{ar}), 7.47 (t, J = 7.7 Hz, 3H, CH_{ar}), 7.58 (dd, J =

6.0, 7.6 Hz, 1H, CH_{ar}), 7.76 – 7.88 (m, 3H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 29.9 (CH_2Br), 32.3 (CH_2), 65.6 (OCH_2), 115.2, 119.2, 123.1, 128.3, 129.4, 130.1, 132.5, 137.6, 139.0, 158.7 (12C, C-Ar), 196.4 (CO). ESI-MS calcd. for $C_{16}H_{15}BrO_2$ $[M+Na]^+$: 341.02; found 341.00 /z. Anal. Calcd: C 60.21, H 4.74; found C 60.02, H 4.89.

(3-(4-bromobutoxy)phenyl)(phenyl)methanone (12b). 3-Hydroxybenzophenone (**11**, 100 mg, 0.5 mmol, 1.0 eq) was dissolved in MeOH (2 ml) and 1,4-dibromobutane (295 µl, 540 mg, 2.5 mmol, 5.0 eq) and potassium carbonate (138 mg, 1.0 mmol, 2.0 eq) were added successively. The reaction mixture was stirred at 50 °C for 5 h. After cooling to r.t., the solvent was evaporated and ethyl acetate was added. Then, the organic layer was washed with brine and water, dried over Na_2SO_4, filtered and the solvents were removed under reduced pressure. The crude product was purified by chromatography on silica gel (petrol ether/ ethyl acetate 4:1) to afford **12b** (132 mg, 78%). ^1H NMR (500 MHz, $CDCl_3$) δ 1.96 (dq, *J* = 6.2, 10.2 Hz, 2H, CH_2), 2.02 – 2.12 (m, 2H, CH_2), 3.48 (t, *J* = 6.6 Hz, 2H, CH_2Br), 4.04 (t, *J* = 6.1 Hz, 2H, OCH_2), 7.11 (ddd, *J* = 1.3, 2.4, 7.8 Hz, 1H, CH_{ar}), 7.30 – 7.39 (m, 3H, CH_{ar}), 7.47 (t, *J* = 7.7 Hz, 2H, CH_{ar}), 7.58 (t, *J* = 7.4 Hz, 1H, CH_{ar}), 7.75 – 7.84 (m, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 27.9 (CH_2), 29.5 (CH_2), 33.4 (CH_2Br), 67.2 (OCH_2), 115.0, 119.3, 123.0, 128.3, 129.3, 130.1, 132.5, 137.7, 139.0, 158.9 (12C, C-Ar), 196.5 (CO). ESI-MS calcd. for $C_{17}H_{17}BrO_2$ $[M+Na]^+$: 355.09; found m/z 354.97. Anal. Calcd: C 61.28, H 5.14; found: C 61.34, H 5.21.

(3-(3-azidopropoxy)phenyl)(phenyl)methanone (13a). Compound **12a** (119 mg, 0.37 mmol, 1.0 eq) was dissolved in DMF (1 mL) and sodium azide (30 mg, 0.46 mmol, 1.2 eq) was added. The reaction mixture was stirred for 24 h at 65 °C. After removal of the solvent under high vacuum, the pure product **13a** (74 mg, 70%) was obtained after chromatography on silica gel (petrol ether/ ethyl acetate 5:1) as colorless oil. IR (NaCl) ν 2097 (N_3), 1656 (CO) cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.96 – 2.20 (m, 2H, CH_2), 3.53 (t, *J* = 6.6 Hz, 2H, CH_2N_3), 4.11 (t, *J* = 5.9 Hz, 2H, OCH_2), 7.10 – 7.15 (m, 1H, CH_{ar}), 7.30 – 7.40 (m, 3H, CH_{ar}), 7.49 (t, *J* = 7.7 Hz, 2H, CH_{ar}), 7.59 (t, *J* = 7.4 Hz, 1H, CH_{ar}), 7.75 – 7.88 (m, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 28.7 (CH_2), 48.2 (CH_2N_3), 64.8 (OCH_2), 115.0, 119.2, 123.1, 128.3, 129.3, 130.0, 132.5, 137.6, 139.0, 158.7 (12C, C-Ar), 196.4 (CO). ESI-MS calcd. for $C_{16}H_{15}N_3O_2$ $[M+Na]^+$: 304.11; found m/z 304.11. Analysis calcd. C 69.14, H 5.80, N 14.23; found C 69.10, H 6.07, N 13.98.

[3-(4-azidobutoxy)phenyl](phenyl)methanone (13b). Compound **12b** (130 mg, 0.39 mmol, 1.0 eq) was dissolved in DMF (1 mL) and sodium azide (30 mg, 0.46 mmol, 1.2 eq) was added. The reaction mixture was stirred for 24 h at 65 °C. Afterwards, the solvent was removed under high vacuum and the crude product was purified by chromatography on silica gel (petrol ether/ ethyl acetate 5:1) to yield **13b** as colorless oil (112 mg, 97%). IR (NaCl) ν 2096 (N_3), 1655 (CO) cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.76 – 1.85 (m, 2H, CH_2), 1.85 – 1.94 (m, 2H), 3.37 (t, *J* = 6.7 Hz, 2H, CH_2N_3), 4.04 (t, *J* = 6.0 Hz, 2H, OCH_2), 7.09 – 7.17 (m, 1H, CH_{ar}), 7.32 - 7.39 (m, 3H, CH_{ar}), 7.48 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.59 (t, *J* = 7.4 Hz, 1H, CH_{ar}), 7.80 (d, *J* = 7.2 Hz, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 25.7 (CH_2), 26.5 (CH_2), 51.2 (CH_2N_3), 67.4 (OCH_2), 114.9, 119.3, 122.9, 128.3, 129.3, 130.0, 132.4, 137.6, 138.9, 158.9 (12C, C-Ar), 196.5 (CO). ESI-MS calcd. for $C_{17}H_{17}N_3O_2$ $[M+Na]^+$: 318.11; found m/z 317.92. Analysis caldc. C 69.14, H 5.80, N 14.23; found C 69.10, H 6.07, N 13.98.

Methyl [2-propynyl 5-acetamido-4,7,8-tri-*O*-acetyl-3,5,9-trideoxy-9-(4-phenyl) benzamido -D -glycero-α-D -galacto-2-nonulopyranosid]onate (14a). Compound **2** (206 mg, 0.31 mmol, 1.0 eq) was reacted with propargyl alcohol (54 µL, 51 mg, 0.9 mmol, 3.0 eq) according to procedure A, to yield **14a** (142 mg, 68%) after chromatography on silica gel (5% gradient of *i*PrOH in PE/DCM 2/1). $[\alpha]^{20}_D$ 2.9 (*c* 0.1, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.89 (s, 3H, NHAc), 1.96 – 2.04 (m, 4H, OAc, H-3a), 2.11, 2.26 (2s, 6H, OAc), 2.38 – 2.52 (m, 2H, H-3b, C≡CH), 2.98 (ddd, *J* = 4.4, 7.4, 15.2 Hz, 1H, H-9a), 3.81 (s, 3H, OMe), 3.96 (dd, *J* = 1.3, 10.6 Hz, 1H, H-6), 4.17 (dd, *J* = 2.4, 13.5 Hz, 2H, $OCH_2C{\equiv}CH$), 4.19 – 4.27 (m, 1H, H-5), 4.54 (ddd, *J* = 3.2, 8.9, 15.2 Hz, 1H, H-9b), 5.20 – 5.33 (m, 3H, H-4, H-7, H-8), 5.44 (d, *J* = 10.2 Hz, 1H, N*H*Ac), 7.15 (dd, *J* = 4.0, 8.8 Hz, 1H, NH), 7.39 (t, *J* = 7.3 Hz, 1H, CH_{ar}), 7.46 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.61 (d, *J* = 7.5 Hz, 2H, CH_{ar}), 7.66 (d, *J* = 8.1 Hz, 2H, CH_{ar}), 7.90 (d, *J* = 8.2 Hz, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 21.1, 21.2, 21.3 (3 OAc), 23.2 (NHAc), 37.2 (C-3), 37.8 (C-9), 49.5 (C-5), 51.8 ($OCH_2C{\equiv}CH$), 53.0 (OMe), 68.0, 68.7, 70.0 (C-4, C-7, C-8), 71.1 (C-6), 72.4 ($C{\equiv}CH$), 78.8 ($C{\equiv}CH$), 98.4 (C-2), 127.2, 127.3, 127.5, 127.6, 128.9, 132.9, 140.0, 144.4 (12C, C-Ar), 166.9, 167.1, 170.4, 170.5, 171.1, 172.3 (6 CO). ESI-MS calcd. for $C_{34}H_{38}N_2O_{12}$ $[M+Na]^+$: 689.23; found m/z 689.39.

Methyl [3-butynyl 5-acetamido-4,7,8-tri-*O*-acetyl-3,5,9-trideoxy-9- (4-phenyl) benzamido-D -glycero-α-D -galacto-2-nonulopyranosid]onate (14b). Compound **2** (217

mg, 0.33 mmol, 1.0 eq) was reacted with but-3-yn-1-ol (75 µl, 69 mg, 0.99 mmol, 3.0 eq) according to procedure A, to yield **14b** (193 mg, 81%) after chromatography on silica gel (5% gradient of *i*PrOH in PE/DCM 2/1). $[\alpha]^{20}_{D}$ -12.5 (*c* 0.9, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.89 (s, 3H, NHAc), 1.97 (m, 1H, H-3a), 2.03 (s, 3H, OAc), 2.05 (t, *J* = 2.7 Hz, 1H, C≡CH), 2.10, 2.27 (2s, 6H, 2 OAc), 2.39 – 2.49 (m, 2H, CH_2), 2.62 (dd, *J* = 4.6, 12.8 Hz, 1H, H-3b), 2.98 (ddd, *J* = 4.4, 7.3, 15.1 Hz, 1H, H-9a), 3.39 – 3.52 (m, 2H, OCH_2), 3.81 (s, 3H, OMe), 4.01 – 4.07 (m, 1H, H-6), 4.16 – 4.26 (m, 1H, H-5), 4.54 (ddd, *J* = 3.4, 8.9, 15.1 Hz, 1H, H-9b), 4.84 (ddd, *J* = 4.7, 10.4, 12.3 Hz, 1H, H-4), 5.20 – 5.25 (m, 1H, H-7), 5.26 – 5.33 (m, 1H, H-8), 5.36 (d, *J* = 10.1 Hz, 1H, NHAc), 7.17 (dd, *J* = 4.0, 8.8 Hz, 1H, NH), 7.36 – 7.42 (m, 1H, CH_{ar}), 7.46 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.59 – 7.64 (m, 2H, CH_{ar}), 7.66 (d, *J* = 8.4 Hz, 2H, CH_{ar}), 7.90 (d, *J* = 8.4 Hz, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 19.6 (CH_2), 20.9, 21.2, 21.3 (3 OAc), 23.2 (NHAc), 37.5 (C-3), 37.8 (C-9), 49.6 (C-5), 52.9 (OMe), 61.6 (O*C*H_2), 68.1, 68.8, 69.4 (C-4, C-7, C-8), 70.1 (C-6), 70.2 (C≡*C*H), 80.9 (*C*≡CH), 98.4 (C-2), 127.2, 127.3, 127.6, 128.0, 128.9, 132.9, 140.0, 144.3 (12C, C-Ar), 167.1, 167.4, 170.3, 170.4, 171.2, 172.3 (6 CO). HRMS calcd. for $C_{35}H_{40}N_2O_{12}$ $[M+Na]^+$: 703.2479; found m/z 703.2474.

Methyl [4-pentynyl 5-acetamido-4,7,8-tri-*O*-acetyl-3,5,9-trideoxy-9-(4-phenyl) benzamido-D -glycero-α-D -galacto-2-nonulopyranosid]onate (14c). Compound **2** (193 mg, 0.29 mmol, 1.0 eq) was reacted with pent-4-yn-1-ol (90 µl, 82 mg, 1.0 mmol, 3.4 eq) according to procedure A, to yield **14c** 140 mg, 70%) after chromatography on silica gel (5% gradient of *i*PrOH in PE/DCM 2/1).^{1}H NMR (500 MHz, $CDCl_3$) δ 1.62 – 1.82 (m, 2H, CH_2), 1.88 (s, 3H, NHAc), 1.90 – 1.94 (m, 1H, H-3a), 1.93 – 1.97 (m, 1H, C≡CH), 2.02, 2.12, 2.21 (3s, 9H, 3 OAc), 2.25 – 2.31 (m, 2H, CH_2), 2.58 (dd, *J* = 4.6, 12.7 Hz, 1H, H-3b), 3.09 – 3.16 (m, 1H, H-9a), 3.25 – 3.42 (m, 2H, O*C*H_2), 3.76 (s, 3H, OMe), 3.90 – 3.99 (m, 1H, H-6), 4.09 – 4.24 (m, 1H, H-5), 4.44 (d, *J* = 12.1 Hz, 1H, H-9b), 4.77 – 4.85 (m, 1H, H-4), 5.18 – 5.23 (m, 1H, H-8), 5.24 – 5.29 (m, 1H, H-7), 5.38 – 5.46 (m, 1H, N*H*Ac), 7.24 (dd, *J* = 4.3, 7.6 Hz, 1H, NH), 7.37 (t, *J* = 7.3 Hz, 1H, CH_{ar}), 7.45 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.59 (d, *J* = 7.7 Hz, 2H, CH_{ar}), 7.64 (dd, *J* = 2.4, 8.3 Hz, 2H, CH_{ar}), 7.88 (dd, *J* = 3.4, 8.2 Hz, 2H, CH_{ar}). ^{13}C NMR ($CDCl_3$) δ 15.0 (CH_2), 20.8, 20.9, 21.1 (3 OAc), 23.1 (NHAc), 28.6 (CH_2), 37.5 (C-3), 38.4 (C-9), 49.4 (C-5), 52.8 (OMe), 62.1 (OCH_2), 68.0 (C≡*C*H), 68.5, 68.9, 69.4 (C-4, C-7, C-8), 70.9 (C-6), 83.8 (*C*≡CH), 98.6 (C-2), 127.1, 127.2, 127.5, 127.6, 128.0, 128.9, 133.0,

140.0, 144.3 (12C, C-Ar), 167.2, 167.7, 168.3, 170.4, 171.1, 171.9 (6 CO). ESI-MS calcd. for $C_{36}H_{42}N_2O_{12}$ $[M+Na]^+$: 717.26; found m/z 717.38.

Sodium [2-propynyl 5-acetamido -3,5,9-trideoxy-9-(4-phenyl)benzamido-D-glycero-α-D -galacto-2-nonulopyranosid]onate (15a). Compound **15a** was obtained from **14a** (142 mg, 0.21 mmol) according to procedure B as white solid after LC-MS purification (94 mg, 80%). $[\alpha]^{20}_D$ -6.3 (*c* 0.83, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.69 (dd, *J* = 11.3, 12.9 Hz, 1H, H-3a), 1.98 (s, 3H, NHAc), 2.39 (dd, *J* = 4.9, 13.0 Hz, 1H, H-3b), 2.79 (dd, *J* = 2.3, 3.7 Hz, 1H, C≡CH), 3.47 – 3.51 (m, 1H, H-7), 3.52 – 3.63 (m, 1H, H-9a), 3.77 – 3.86 (m, 1H, H-9b), 3.88 – 3.94 (m, 2H, H-5, H-6), 3.96 (ddd, *J* = 4.1, 7.5, 14.5 Hz, 1H, H-8), 4.00 – 4.07 (m, 1H, H-4), 4.09, 4.41 (dd, *J* = 3.3, 15.7 Hz, 2H, OC*H*$_2$), 7.38 (td, *J* = 1.2, 7.3 Hz, 1H, CH_{ar}), 7.46 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.66 (d, *J* = 7.8 Hz, 2H, CH_{ar}), 7.69 – 7.76 (m, 2H, CH_{ar}), 7.89 – 7.96 (m, 2H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 22.7 (NHAc), 41.5 (C-3), 45.9 (C-9), 52.7 (O*C*H$_2$), 53.6 (C-5), 67.6 (C-4), 70.6 (C-8), 71.7 (C-7), 72.8 (C-6), 75.6 (C≡*C*H), 80.2 (*C*≡CH), 98.1 (C-2), 128.0, 128.1, 128.9, 129.0, 129.1, 130.0, 134.3, 141.3, 145.8 (12C, C-Ar), 170.9, 174.9 (2 CO). ESI-MS calcd. for $C_{27}H_{29}N_2NaO_9$ $[M+H]^+$: 549.18; found m/z 549.19.

Sodium [3-butynyl 5-acetamido -3,5,9-trideoxy-9-(4-phenyl)benzamido -D-glycero-α-D -galacto-2-nonulopyranosid]onate (15b). Compound **15b** was obtained from **14b** (183 mg, 0.27 mmol) according to procedure B as white solid after LC-MS purification (62 mg, 40%). $[\alpha]^{20}_D$ -8.1(*c* 0.9, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.68 (dd, *J* = 11.5, 12.6 Hz, 1H, H-3a), 1.98 (s, 3H, NHAc), 2.24 (t, *J* = 2.6 Hz, 1H, C≡CH), 2.38 (dd, *J* = 4.9, 12.9 Hz, 1H, H-3b), 2.46 (t, *J* = 7.0 Hz, 2H, CH_2), 3.41 – 3.51 (m, 2H, H-6, OCH_2), 3.58 (dd, *J* = 6.8, 13.9 Hz, 1H, H-9a), 3.79 – 3.86 (m, 2H, H-9b, OCH_2), 3.90 (d, *J* = 9.9 Hz, 1H, H-5), 3.97 (m, 2H, H-7, H-8), 4.01 – 4.09 (m, 1H, H-4), 7.38 (t, *J* = 7.4 Hz, 1H, CH_{ar}), 7.46 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.65 (d, *J* = 7.8 Hz, 2H, CH_{ar}), 7.72, 7.92 (d, *J* = 8.3 Hz, 4H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 20.3 (CH_2), 22.7 (NHAc), 41.7 (C-3), 45.8 (C-9), 53.8 (C-5), 62.9 (OCH_2), 67.6 (C-4), 70.6, 70.7 (2C, C-7, C-8), 71.7 (C-6), 72.5 (C≡*C*H), 81.7 (*C*≡CH), 100.3 (C-2), 128.0, 128.1, 129.0, 130.0, 134.2, 141.2, 145.7 (12C, C-Ar), 170.9, 174.8 (2 CO). HRMS-MS calcd. for $C_{28}H_{32}N_2O_9$ $[M+Na]^+$: 563.2007; found 563.2001 m/z.

Sodium [5-pentynyl 5-acetamido -3,5,9-trideoxy-9-(4-phenyl)benzamido -D-glycero-α-D -galacto-2-nonulopyranosid]onate (15c). Compound **15c** was obtained from **14c** (130 mg,

0.19 mmol) according to procedure B as white solid after LC-MS purification (34 mg, 28%). $[\alpha]^{20}_{D}$ -6.1 (*c* 0.85, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.64 – 1.79 (m, 3H, CH_2, H-3a), 1.99 (s, 3H, NHAc), 2.15 (t, *J* = 2.6 Hz, 1H, C≡CH), 2.24 (tt, *J* = 2.4, 7.0 Hz, 2H, CH_2), 2.74 (dd, *J* = 4.5, 12.6 Hz, 1H, H-3b), 3.46 (dd, *J* = 1.3, 8.8 Hz, 1H, H-7), 3.50 – 3.61 (m, 2H, OCH_2, H-9a), 3.63 – 3.80 (m, 3H, H-4, H-5, H-6), 3.83 (dd, *J* = 3.4, 13.7 Hz, 1H, H-9b), 3.88 (dt, *J* = 6.1, 9.3 Hz, 1H, OCH_2), 4.02 – 4.11 (m, 1H, H-8), 7.38 (t, *J* = 7.4 Hz, 1H, CH_{ar}), 7.46 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.63 – 7.69 (m, 2H, CH_{ar}), 7.72, 7.92 (d, *J* = 8.4 Hz, 4H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 15.8 (CH_2), 22.6 (NHAc), 30.1 (CH_2), 42.0 (C-3), 44.7 (C-9), 54.0 (C-5), 63.8 (OCH_2), 69.0 (C-4), 69.7 (C≡*C*H), 71.2 (C-8), 72.3 (C-7), 74.6 (C-6), 84.6 (*C*≡CH), 100.9 (C-2), 128.0, 128.1, 128.9, 129.1, 130.0, 134.4, 141.3, 145.6 (12C, C-Ar), 170.2, 175.3 (2 CO). HRMS calcd. for $C_{29}H_{33}N_2NaO_9$ $[M+Na]^+$: 599.1982; found m/z 599.1984.

Sodium [(1-(2-(3-benzoylphenoxy)ethyl)-1H-1,2,3-triazol-4-yl)methyl) 5-acetamido -3,5,9-trideoxy-9-(4-phenyl)benzamido-D-glycero-α-D-galacto-2-nonulopyranosid] onate (16a). Compound **15a** (7 mg, 13 µmol, 1.0 eq) was reacted with **10** (5 mg, 20 µmol, 1.5 eq) according to procedure C to yield **16a** (1.3 mg, 12%) after LC-MS purification. $[\alpha]^{20}_{D}$ 11.9 (*c* 0.13, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.69 (t, *J* = 11.3 Hz, 1H, H-3a), 1.94 (s, 3H, NHAc), 2.39 – 2.41 (m, 1H, H-3b), 3.46 (d, *J* = 9.5 Hz, 1H, H-7), 3.50 – 3.62 (m, 1H, H-9a), 3.82 – 3.91 (m, 1H, H-9b), 3.91 – 4.02 (m, 2H, H-4, H-5), 4.06 – 4.08 (m, 2H, H-6, H-8), 4.47 (t, *J* = 4.8 Hz, 2H, H-2''), 4.54, 4.75 (A, B of AB, *J* = 11.2 Hz, 2H, H-1'), 4.83 (t, *J* = 4.8 Hz, 2H, H-1'') 7.22 (dd, *J* = 2.2, 8.1 Hz, 1H, CH_{ar}), 7.26 – 7.34 (m, 2H, CH_{ar}), 7.34 – 7.55 (m, 7H, CH_{ar}), 7.63 (dd, *J* = 7.5, 16.7 Hz, 3H, CH_{ar}), 7.69 (d, *J* = 8.3 Hz, 2H, CH_{ar}), 7.74 (d, *J* = 7.4 Hz, 2H, CH_{ar}), 7.91 (d, *J* = 8.3 Hz, 2H, CH_{ar}), 7.97 (d, *J* = 7.9, 1H, 5-NH), 8.16 (s, 1H, CH_{ar}), 8.40 (t, *J* = 5.3 Hz, 1H, 9-NH). ^{13}C NMR (CD_3OD) δ 22.9 (NHAc), 43.5 (C-3), 45.6 (C-9), 50.6 (C-1''), 53.7 (C-5), 57.9 (C-1'), 67.8 (C-2''), 68.3 (C-4), 70.6, 72.0, 72.3 (C-6, C-7, C-8), 116.4, 120.4, 124.4, 128.0, 128.1, 129.0, 129.1, 129.5, 130.0, 130.7, 131.0, 133.9 (26C, C-Ar). ESI-MS calcd. for $C_{42}H_{43}N_5O_{11}$ $[M-H]^-$: 792.28; found m/z 792.39.

Sodium [(1-(3-(3-benzoylphenoxy)propyl)-1H-1,2,3-triazol-4-yl)methyl) 5-acetamido -3,5,9-trideoxy-9-(4-phenyl)benzamido-D-glycero-α-D-galacto-2-nonulopyranosid] onate (16b). Compound **15a** (6 mg, 11 µmol, 1.0 eq) was reacted with **13a** (10 mg, 36 µmol, 3.2 eq) according to procedure C to yield **16b** (4.9 mg, 54%) after LC-MS purification. $[\alpha]^{20}_{D}$ 9.4 (*c* 0.2, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.70 (t, *J* = 11.3 Hz, 1H, H-3a), 1.96 (s, 3H,

NHAc), 2.34 – 2.46 (m, 3H, H-3b, H-2''), 3.47 (d, *J* = 9.3 Hz, 1H, H-7), 3.56 (dd, *J* = 6.9, 13.6 Hz, 1H, H-9a), 3.87 (dd, *J* = 2.8, 13.8 Hz, 1H, H-9b), 3.92 – 4.02 (m, 2H, H-4, H-5), 4.04 – 4.13 (m, 4H, H-3'', H-6, H-8), 4.52 (A of AB, *J* = 11.0 Hz, 1H, H-1'a), 4.63 (t, *J* = 6.8 Hz, 2H, H-1''), 4.78 (B, of AB, *J* = 11.0 Hz, 1H, H-1'b), 7.19 (d, *J* = 8.4 Hz, 1H, CH_{ar}), 7.28 (m, 2H, CH_{ar}), 7.39 (m, 2H, CH_{ar}), 7.45 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.51 (t, *J* = 7.7 Hz, 2H, CH_{ar}), 7.63 (m, 3H, CH_{ar}), 7.70 (d, *J* = 8.3 Hz, 2H, CH_{ar}), 7.75 (d, *J* = 7.3 Hz, 2H, CH_{ar}), 7.92 (d, *J* = 8.3 Hz, 2H, CH_{ar}), 8.05 (s, 2H, 5-NH, CH_{ar}), 8.44 (t, *J* = 5.6 Hz, 1H, 9-NH). ^{13}C NMR (CD_3OD) δ 22.8 (NHAc), 31.0 (C-2''), 41.7 (C-3), 45.6 (C-9), 48.4 (C-1''), 53.8 (C-5), 57.5 (C-1'), 65.9 (C-3''), 68.0 (C-4), 70.5 (C-8), 72.1 (C-7), 72.3 (C-6), 98.0 (C-2), 116.2, 120.4, 124.0, 128.0, 128.1, 129.0, 129.1, 129.5, 130.0, 130.6, 131.0, 133.9, 134.3, 138.8, 140.1, 141.3, 145.7, 160.1 (19C, C-Ar), 170.3, 170.7, 174.4, 198.3 (4 CO). ESI-MS calcd. for $C_{43}H_{45}N_5O_{11}$ $[M-H]^-$: 806.30; found m/z 806.27.

Sodium [(1-(4-(3-benzoylphenoxy)butyl)-1H-1,2,3-triazol-4-yl)methyl) 5-acetamido -9-(4-phenyl)-3,5,9-trideoxy-D-glycero-α-D -galacto-2-nonulopyranosid] onate (16c). Compound **15a** (7 mg, 13 μmol, 1.0 eq) was reacted with **13b** (10 mg, 34 μmol, 2.6 eq) according to procedure C to yield **16c** (2.7 mg, 25%) after LC-MS purification. $[\alpha]^{20}_D$ 11.8 (*c* 0.3,MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.70 (t, *J* = 11.0 Hz, 1H, H-3a), 1.81 (dt, *J* = 6.2, 12.9 Hz, 2H, H-3''), 1.96 (s, 3H, NHAc), 2.06 – 2.15 (m, 2H, H-2''), 2.39 (d, *J* = 11.2 Hz, 1H, H-3b), 3.48 (d, *J* = 9.3 Hz, 1H, H-7), 3.52 – 3.60 (m, 1H, H-9a), 3.81 – 3.91 (m, 1H, H-9b), 3.96 (m, 2H, H-4, H-5), 4.05 (m, 4H, H-4'', H-6, H-8), 4.45 – 4.58 (m, 3H, H-1'a, H-1''), 4.78 (B of AB, *J* = 11.0 Hz, 1H, H-1'b), 7.16 – 7.22 (m, 1H, CH_{ar}), 7.27 (d, *J* = 7.8 Hz, 2H, CH_{ar}), 7.32 – 7.42 (m, 2H, CH_{ar}), 7.45 (t, *J* = 7.5 Hz, 2H, CH_{ar}), 7.51 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.59 – 7.67 (m, 3H, CH_{ar}), 7.70 (d, *J* = 8.2 Hz, 2H, CH_{ar}), 7.75 (d, *J* = 7.6 Hz, 2H, CH_{ar}), 7.92 (d, *J* = 8.2 Hz, 2H, CH_{ar}), 8.04 (s, 2H, 5-NH, CH_{ar}), 8.45 (t, *J* = 5.5 Hz, 1H, 9-NH). ^{13}C NMR (CD_3OD) δ 22.8 (NHAc), 27.3 (C-3''), 28.1 (C-2''), 41.6 (C-3), 45.6 (C-9), 51.0 (C-1''), 53.8 (C-5), 57.6 (C-1'), 67.9 (C-4), 68.4 (C-4''), 70.6 (C-8), 72.1 (C-7), 72.3 (C-6), 116.1, 120.3, 123.7, 128.0, 128.1, 129.0, 129.1, 129.5, 130.0, 130.6, 131.0, 133.9, 140.1, 141.3, 145.7, 160.4 (19C, C-Ar), 174.4, 176.1, 198.4 (4C, CO). ESI-MS calcd. for $C_{44}H_{47}N_5O_{11}$ $[M-H]^-$: 820.32; found m/z 820.42.

Sodium [(2-(1-(2-(3-benzoylphenoxy)ethyl)-1H-1,2,3-triazol-4-yl)ethyl) 5-acetamido -9-(4- phenyl)benzamido-3,5,9-trideoxy-D-glycero-α-D -galacto-2-nonulopyranosid] onate

(16d). Compound **15b** (5 mg, 9 µmol, 1.0 eq) was reacted with **10** (3.5 mg, 13 µmol, 1.5 eq) according to procedure C to yield **16d** (3.2 mg, 43%) after LC-MS purification. $[\alpha]^{20}_{D}$ – 6.4 (*c* 0.12, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.65 (t, *J* = 12.1 Hz, 1H, H-3a), 1.99 (s, 3H, NHAc), 2.77 (dd, *J* = 2.2, 12.8 Hz, 1H, H-3b), 2.94 (s, 2H, H-2'), 3.45 (d, *J* = 7.9 Hz, 1H, H-7), 3.50 (dd, *J* = 7.6, 13.5 Hz, 1H, H-9a), 3.62 – 3.90 (m, 5H, H-4, H-5, H-6, H-9b, H-1'a), 3.96 – 4.18 (m, 2H, H-8, H-1'b), 4.44, 4.76 (2t, *J* = 4.3 Hz, 4H, H-1'', H-2''), 7.18 (d, *J* = 8.3 Hz, 1H, CH_{ar}), 7.27 (d, *J* = 7.3 Hz, 2H, CH_{ar}), 7.33 – 7.41 (m, 2H, CH_{ar}), 7.45 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.50 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.63 (d, *J* = 7.6 Hz, 3H, CH_{ar}), 7.67 (d, *J* = 8.1 Hz, 2H, CH_{ar}), 7.73 (d, *J* = 7.8 Hz, 2H, CH_{ar}), 7.88 (d, *J* = 8.1 Hz, 2H, CH_{ar}), 8.06 (s, 1H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 22.3 (NHAc), 28.0 (C-2'), 42.0 (C-3), 44.6 (C-9), 50.6 (C-1''), 53.7 (C-5), 63.6 (C-1'), 67.5 (C-2''), 68.9 (C-4), 70.9 (C-8), 72.2 (C-7), 74.3 (C-6), 102.7, 109.9, 116.0, 120.3, 124.0, 127.7, 127.8, 128.7, 128.8, 129.2, 129.7, 130.5, 130.8, 133.6 (26C, C-Ar), 173.7, 198.0 (3C, CO). ESI-MS calcd. for $C_{43}H_{45}N_5O_{11}$ $[M-H]^-$: 806.30; found m/z 806.34.

Sodium [(2-(1-(3-(3-benzoylphenoxy)propyl])-1H-1,2,3-triazol-4-yl)ethyl) 5-acetamido -9-(4-phenyl)benzamido-3,5,9-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosid] onate (16e). Compound **15b** (5 mg, 9 µmol, 1.0 eq) was reacted with **13a** (3.6 mg, 13 µmol, 1.5 eq) according to procedure C to yield **16e** (3.4 mg, 47%) after LC-MS purification. $[\alpha]^{20}_{D}$ – 8.3 (*c* 0.3, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.59 (t, *J* = 11.4 Hz, 1H, H-3a), 2.01 (s, 3H, NHAc), 2.26 – 2.43 (m, 2H, H-2''), 2.76 – 2.87 (m, 1H, H-3b), 2.92 (d, *J* = 2.5 Hz, 2H, H-2'), 3.41 – 3.53 (m, 2H, H-7, H-9a), 3.61 – 3.79 (m, 4H, H-4, H-5, H-6, H-1'a), 3.84 (dd, *J* = 2.6, 13.4 Hz, 1H, H-9b), 3.97 – 4.11 (m, 4H, H-8, H-1'b, H-3''), 4.55 (t, *J* = 6.8 Hz, 2H, H-1''), 7.15 (dd, *J* = 2.0, 8.1 Hz, 1H, CH_{ar}), 7.23 (s, 1H, CH_{ar}), 7.27 (d, *J* = 7.6 Hz, 1H, CH_{ar}), 7.32 – 7.41 (m, 2H, CH_{ar}), 7.43 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.51 (t, *J* = 7.7 Hz, 2H, CH_{ar}), 7.63 (t, *J* = 6.9 Hz, 3H, CH_{ar}), 7.67 (d, *J* = 8.2 Hz, 2H, CH_{ar}), 7.74 (d, *J* = 7.6 Hz, 2H, CH_{ar}), 7.88 (d, *J* = 8.2 Hz, 2H, CH_{ar}), 8.04 (s, 1H, CH_{ar}), 8.21 (s, 1H, 5-NH), 8.27 (t, *J* = 4.9 Hz, 1H, 9-NH). ^{13}C NMR (CD_3OD) δ 22.6 (NHAc), 27.4 (C-2'), 31.0 (C-2''), 42.7 (C-3), 44.7 (C-9), 48.4 (C-1''), 54.1 (C-5), 63.8 (C-1'), 66.1 (C-3''), 69.5 (C-4), 71.3 (C-8), 72.7 (C-7), 74.4 (C-6), 116.5, 120.2, 123.8, 124.6, 128.0, 128.1, 129.0, 129.1, 129.5, 130.0, 130.7, 131.0, 133.8, 140.1, 141.3, 145.5 (26C, C-Ar), 160.1, 169.9, 175.5, 198.3 (4 CO). ESI-MS calcd. for $C_{44}H_{47}N_5O_{11}$ $[M-H]^-$: 820.32; found m/z 820.41.

Sodium [(2-(1-(4-(3-benzoylphenoxy)butyl)-1H-1,2,3-triazol-4-yl)ethyl) 5-acetamido -9-(4-phenyl)benzamido-3,5,9-trideoxy-D-glycero-α-D -galacto-2-nonulopyranosid] onate (16f). Compound **15b** (5 mg, 9 µmol, 1.0 eq) was reacted with **13b** (3.8 mg, 13 µmol, 1.5 eq) according to procedure C to yield **16f** (3.1 mg, 42%) after LC-MS purification. $[\alpha]^{20}_{D}$ – 8.9 (*c* 0.25, MeOH). ^{1}H NMR (500 MHz, CD_3CN) δ 1.58 (t, *J* = 11.9 Hz, 1H, H-3a), 1.65 – 1.77 (m, 2H, H-3''), 1.88 – 2.02 (m, 5H, H-2'', NHAc), 2.45 – 2.79 (m, 1H, H-3b), 2.81 – 2.91 (m, 2H, H-2'), 3.39 – 3.54 (m, 3H, H-6, H-7, H-9a), 3.59 (t, *J* = 8.8 Hz, 1H, H-5), 3.62 – 3.82 (m, 3H,H-4, H-9a, H-1'a), 3.84 – 3.91 (m, 1H, H-8), 3.97 (t, *J* = 5.9 Hz, 3H, H-1'b, H-4''), 4.33 (t, *J* = 6.7 Hz, 2H, H-1''), 7.13 (d, *J* = 8.2 Hz, 1H, CH_{ar}), 7.23 (s, 1H, CH_{ar}), 7.26 (d, *J* = 7.5 Hz, 1H, CH_{ar}), 7.35 – 7.41 (m, 2H, CH_{ar}), 7.45 (t, *J* = 7.5 Hz, 2H, CH_{ar}), 7.50 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.60 – 7.66 (m, 3H, CH_{ar}), 7.68 (d, *J* = 7.9 Hz, 2H, CH_{ar}), 7.74 (d, *J* = 7.5 Hz, 3H, CH_{ar}), 7.85 (d, *J* = 7.8 Hz, 2H, CH_{ar}). ^{13}C NMR (CD_3CN) δ 22.9 (NHAc), 26.0 (C-3''), 26.9 (2C, C-2', C-2''), 41.4 (C-3), 44.5 (C-9), 50.5 (C-1''), 53.5 (C-5), 63.3 (C-1'), 67.8 (C-4''), 68.3 (C-4), 71.7 (2C, C-7, C-8), 74.1 (C-6), 116.0, 120.0, 123.3, 127.9, 128.0, 128.7, 129.0, 129.4, 130.0, 130.5, 130.8, 133.6 (26C, CO), 173 (4C, CO). ESI-MS calcd. for $C_{45}H_{49}N_5O_{11}$ $[M-H]^-$: 835.34; found m/z 834.49.

Sodium [(2-(1-(2-(3-benzoylphenoxy)ethyl)-1H-1,2,3-triazol-4-yl)ethoxy) 5-acetamido -9-(4-phenyl)benzamido-3,5,9-trideoxy-D-glycero-α-D -galacto-2-nonulopyranosid] onate (16g). Compound **15c** (xx mg, xx µmol, 1.0 eq) was reacted with **10** (xx mg, xx µmol, 1.5 eq) according to procedure C to yield **16g** (1.4 mg, --%) after LC-MS purification. $[\alpha]^{20}_{D}$ – 5.9 (*c* 0.13, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.64 (t, *J* = 11.6 Hz, 1H, H-3a), 1.76 – 1.91 (m, 2H, H-2'), 2.01 (s, 3H, NHAc), 2.71 – 2.87 (m, 3H, H-3b, H-3'), 3.46 (dd, *J* = 6.2, 15.8 Hz, 3H, H-7, H-9a, H-1'a), 3.64 (d, *J* = 9.1 Hz, 1H, H-6), 3.68 – 3.75 (m, 2H, H-4, H-5), 3.80 (dt, *J* = 6.7, 8.9 Hz, 1H, H-1'b), 3.87 (dt, *J* = 3.9, 13.6 Hz, 1H, H-9b), 4.01 (td, *J* = 3.2, 8.4 Hz, 1H, H-8), 4.42 (t, *J* = 5.1 Hz, 2H, H-2''), 4.77 (dd, *J* = 4.6, 7.9 Hz, 2H, H-1''), 7.17 (dd, *J* = 2.2, 8.2 Hz, 1H, CH_{ar}), 7.24 (s, 1H), 7.27 (d, *J* = 7.6 Hz, 1H, CH_{ar}), 7.37 (q, *J* = 8.0 Hz, 2H, CH_{ar}), 7.44 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.50 (t, *J* = 7.7 Hz, 2H, CH_{ar}), 7.62 (t, *J* = 7.3 Hz, 3H, CH_{ar}), 7.68 (d, *J* = 8.3 Hz, 2H, CH_{ar}), 7.72 (d, *J* = 7.5 Hz, 2H, CH_{ar}), 7.88 (d, *J* = 8.3 Hz, 2H, CH_{ar}), 7.97 (s, 1H, CH_{ar}), 8.19 (d, *J* = 7.6 Hz, 1H, 5-NH), 8.25 (t, *J* = 5.2 Hz, 1H, 9-NH). ^{13}C NMR (CD_3OD) δ 22.3 (C-3'), 22.6 (NHAc), 30.4 (C-2'), 42.7 (C-3), 44.8 (C-9), 50.8 (C-1''), 54.2 (C-5), 63.3 (C-1'), 67.9 (C-2''), 69.5 (C-4), 71.2 (C-8), 72.7 (C-7), 74.4 (C-6), 103.0 (C-2), 116.4, 120.5, 124.2, 128.0, 128.1, 129.0, 129.1, 129.5, 130.0, 130.7, 131.0,

133.9, 134.4, 138.7, 140.1, 141.3, 145.5 (26C, C-Ar), 175.5 (4C, CO). ESI-MS calcd. for $C_{44}H_{47}N_5O_{11}$ $[M-H]^-$: 820.32; found m/z 820.57

Sodium [(2-(1-(4-(3-benzoylphenoxy)butyl)-1H-1,2,3-triazol-4-yl)ethyl) 5-acetamido -9-(4-phenyl)benzamido-3,5,9-trideoxy-D-glycero-α-D -galacto-2-nonulopyranosid] onate (16h). Compound **15c** (xx mg, xx µmol, 1.0 eq) was reacted with **13b** (xx mg, xx µmol, 1.5 eq) according to procedure C to yield **16h** (3.4 mg, --%) after LC-MS purification. $[\alpha]^{20}_D$ – 7.2 (*c* 0.23, MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 1.69 (t, *J* = 11.7 Hz, 1H, H-3a), 1.75 – 1.82 (m, 2H, H-3''), 1.83 – 1.97 (m, 2H, H-2'), 1.96 (s, 3H, NHAc), 2.04 – 2.14 (m, 2H, H-2''), 2.71 – 2.88 (m, 3H, H-3b, H-3'), 3.43 – 3.56 (m, 3H, H-7, H-9a, H-1'a), 3.66 (d, *J* = 9.9 Hz, 1H, H-6), 3.69 – 3.92 (m, 4H, H-4, H-5, H-9b, H-1'b), 4.01 (t, *J* = 6.1 Hz, 3H, H-8, H-4''), 4.38 – 4.51 (m, 2H, H-1''), 7.18 (dd, *J* = 1.9, 8.1 Hz, 1H, CH_{ar}), 7.25 – 7.32 (m, 2H, CH_{ar}), 7.35 – 7.43 (m, 2H, CH_{ar}), 7.46 (t, *J* = 7.6 Hz, 2H, CH_{ar}), 7.53 (t, *J* = 7.7 Hz, 2H, CH_{ar}), 7.66 (d, *J* = 7.4 Hz, 3H, CH_{ar}), 7.70 (d, *J* = 8.3 Hz, 2H, CH_{ar}), 7.77 (d, *J* = 7.4 Hz, 2H, CH_{ar}), 7.92 (d, *J* = 8.2 Hz, 3H, CH_{ar}), 8.20 (d, *J* = 7.9 Hz, 1H, CH_{ar}), 8.31 (t, *J* = 4.9 Hz, 1H, CH_{ar}). ^{13}C NMR (CD_3OD) δ 22.6 (C-3'), 22.7 (NHAc), 27.3 (C-3''), 28.1 (C-2''), 30.3 (C-2'), 42.1 (C-3), 44.8 (C-9), 51.0 (C-1''), 54.1 (C-5), 63.4 (C-1'), 68.4 (C-4''), 69.3 (C-4), 71.2 (C-8), 72.5 (C-7), 74.5 (C-6), 100.3 (C-2), 116.2, 120.4, 123.6, 128.0, 128.1, 129.0, 129.1, 129.5, 130.0, 130.6, 131.0, 133.8, 134.4, 138.8, 140.1, 141.3, 145.6 (26C, C-Ar), 160.4, 175.4, 198.4 (4C, CO). ESI-MS calcd. for $C_{46}H_{54}N_5O_{11}$ $[M-H]^-$: 848.35; found m/z 848.46.

NMR. Shigemi NMR tubes were used to reduce the sample volume needed for measurement to 250 µl. $CD22_{d1-3}$-Fc protein was diluted from a stock solution of 0.5 mg/ml by a factor of 2 using 99.8% D_2O (Armar Chemicals). Following dilution, the 0.25 mg/ml $CD22_{d1-3}$-Fc was in a solvent of 50% D_2O and 50% H_2O, with 0.01% NaN_3 with a buffer of 5 mM PBS. For all experiments, CD22 was further diluted with PBS to a protein concentration of 5 µmol. For the T1rho experiemts, stock solutions of all mixes were prepared in DMSO at 25 mM and added to the NMR samples containing $CD22_{d1-3}$-Fc, yielding a 300 µM concentration. For the T1rho experiments the pulse sequence was adapted from Hajduk *et al.*[13]

All NMR experiments were carried out at 300 K on a Bruker DRX500 spectrometer, equipped with Z-gradient SEI probe. The pulse sequence used for the selective inversion recovery experiments began with a selective 25 ms I-Burp-1[14] 180-degree pulse applied to

NHAc of compound **6**. The resonance frequency was sufficiently different from the water resonance, thus avoiding complications due to radiation damping.[15] Following the selective inversion pulse, a 1 ms gradient pulse was applied to dephase any residual transverse magnetization. The gradient pulse was followed by a variable delay to allow for the recovery of longitudinal magnetization. The delay was followed by a DPFGSE water suppression sequence to suppress the magnetization from the 50% H_2O.[15]

For each selective inversion recovery time measurement (sT1), 10 experiments were performed. These experiments consisted of increasing delays following the selective inversion pulse and gradient of 0.1 s, 0.25 s, 0.5 s, 0.75 s, 1 s, 1.25 s, 1.5 s, 2.0 s, 2.5 s and 3 s. 64 scans, preceded by 8 dummy scans, were measured for compound **6** (500 µM, reduced by ascorbic acid 5 mM). The NMR data were analyzed using XWINNMR version 3.5 operating on a PC running under Linux OS. The spectra were apodized with an exponential decay function with 2 Hz line broadening. The inversion recovery data, as well as the one-site binding model, were fit using Prism 4 (GraphPad Software Inc., San Diego, USA).

Surface plasmon resonance (SPR) analysis.

The SPR measurements were performed on a Biacore 3000 surface plasmon resonance based optical biosensor (Biacore AB, Sweden). Sensor chips (CM5), immobilization kits, maintenance supply and HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) were purchased from Biacore AB (HBS-EP ready-to-use; degassed and filtered). CM5 chips were preconditioned prior to usage by injecting a series of conditioning solutions. A flow rate of 50 µL/min was used and 2 × 20 µL of 50 mM NaOH, 10 mM HCl, 0.1% SDS and 100 mM H_3PO_4 were injected. The carboxy groups on the CM5 chip were activated for 10 min with a 1:1 mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.1 M 3-(*N*,*N*-dimethylamino)propyl-*N*-ethylcarbodiimide (EDC) at a flow rate of 10 µL/min. Protein A (P6031) was purchased from Sigma. A sample and a reference surface were prepared sequentially or in parallel. For immobilizing protein A, a stock solution (1 mg/mL in 50 mM phosphate buffer, pH 7.0) was diluted in 10 mM sodium acetate, pH 5.0 to obtain a concentration of 30 µg/mL. This solution was then injected over the activated surface for 10 min at a flow rate of 10 µL/min. Protein A densities around 4'000 RU were achieved. Flow cells were blocked with a 10 min injection of 1 M ethanolamine, pH 8.0. For capturing, Fc-$CD22_{d1-3}$ solution (expressed and purified as described[16]) was diluted to a 30-40 µg/mL concentration in NaOAc (pH 5.0). Afterwards, Fc-$CD22_{d1-3}$ was injected at a flow rate of 5 µL/min for 3 min. Using HBS-EP, the surface was equilibrated over night at a flow

rate of 5 µL/min, achieving densities around 3000 to 4000 RU. Tenfold dilution series were freshly prepared in eluent buffer immediately before use (→ **6**, **15a-c**). All binding experiments were conducted at 25 °C at a flow rate of 20 µL/min. The samples were injected over 1 min followed by 1 min dissociation. Each sample was measured with a duplicate of one concentration, using a randomized concentration order. Several buffer samples were injected before the first concentration, and one blank between each concentration, which was used for the double blank referencing during data processing. Double referencing was applied to correct for bulk effects and other systematic artifacts. Data processing and equilibrium binding constant determinations were accomplished with Scrubber (BioLogic Software, Version 1.1g or 2.0c). Kinetic data were simultaneously fit using Scrubber 2.0c.

For the DMSO assay, DMSO (for molecular biology, >99.9%) was purchased from Fluka. The stock solution of the test compounds (→**16a-h**) was prepared in DMSO and was kept in glass vials to eliminate contaminations by *e.g.* softeners. The running buffer was 5% DMSO in HBS-EP. The surface was equilibrated at a flow of 5 µL/min until the baseline was stable. In order to eliminate the influence of DMSO on the signals, a calibration curve was done. Therefore, two solutions were prepared (A = 1 mL running buffer + 50 µL HBS-EP; B = 1 mL running buffer + 1 µL DMSO). Solutions A and B were mixed as indicated in Table 2 and used for calibration. DMSO calibration solutions were injected after 5 blank injections and before the sample solutions. The test compounds were diluted before measuring with HBS-EP to achieve a content of 5% DMSO. The DMSO calibration was accomplished directly in Scrubber© (version 2.0c).

Table 2. Calibration solutions

Calibration	1	2	3	4	5
A (µL)	400	300	200	100	0
B (µL)	0	100	200	300	400

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3. Summary and Outlook.

Siglecs play an important role in carbohydrate-mediated signaling processes and cell-cell interactions. With the exception of MAG, which is expressed on oligodentrocytes in the CNS, they are expressed and involved in the innate immune system. In the recent years, sialic acid mimetics, displaying up to nM affinities, have been intensively investigated by our group and others.

The ganglioside GQ1bα served as starting point for the development of synthetic sialosides for MAG. Intensive SAR studies led to the identification of antagonists with reduced structural complexity and improved binding affinity.

Here, further improvement was achieved by elucidation of iteratively introduced modifications and finally combination in one molecule. The influence of the electron density of the aglycon on the affinity as well as the contribution of modifications in 5-position were determined. Finally, the thermodynamic properties as well as pharmacokinetics were measured in order to gain additional information about the different contributions to binding. Additionally, a homology model of MAG was used to perform molecular modeling studies for a more profound understanding of the binding at the molecular level.

As carbohydrates suffer in general from short half-life times of the protein-ligand complex, we turned our focus on the amelioration of the kinetic profile of our antagonists. On the basis of a small library of 4-modified antagonists, we investigated the influence of various substituents on the half-life times.

Finally, the most promising antagonist was synthesized in larger amounts compounds as it fulfills all pre-requirements for investigation in nerve-outgrowth assays.

Recently, second-site compounds for MAG were reported to bind with enhanced affinity. Hence, the lead was further optimized by incorporation of substituents based on the knowledge gained about the contributions to binding of the sialic acid moiety in precedent studies. As only little is known about the 5'-nitro indole moiety, additionally, antagonists with modifications at the 5'-nitro indole were synthesized and investigated. Thermodynamic measurements revealed further insights beside the binding affinity, and with the help of modeling we could illustrate the specific role of the 5'-nitro substituent.

Furthermore, a combination of the findings obtained during the optimization of the second-site ligands might be of interest to further improve the binding affinity.

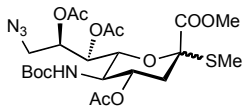
CD22 is currently the most important Siglec with respect to therapeutic applications and many efforts have been undertaken to develop high affinity ligands. Combination of findings reported in literature with in-house screening results yielded nM-binding antagonists. The kinetic properties were found to be improved, what could be due to the increased lipophilicity. Furthermore, pharmacokinetic parameters as pKa, log D and logPe values were measured. Additionally the plasma protein binding was determined, rising potential risks of the antagonists.
With respect to the metabolic stability, nitro-groups are in general not desired. Consequently it remains to elucidate the impact on the binding affinity and replacement by more stable substituents is desirable.

Sialoadhesin and CD22 are expressed in the same physiological compartment and therefore selective targeting might be of interest. As the binding site is conserved in all Siglecs, we discussed different approaches for the achievement of selectivity.
For Sialoadhesin, it might be interesting to apply the second site screening approach, as the modifications in the sialic acid in general did not lead to drastic differences in the binding affinity.

Finally, the library of synthetic sialosides might serve as a starting point for the development of antagonists for other therapeutic important Siglecs such as CD33.

4. Formula index.

-Chapter 2.1.2-



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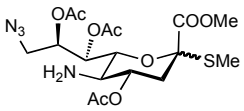

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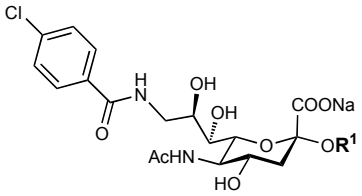

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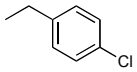
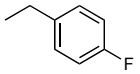
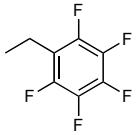
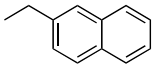
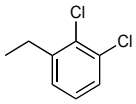
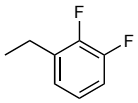
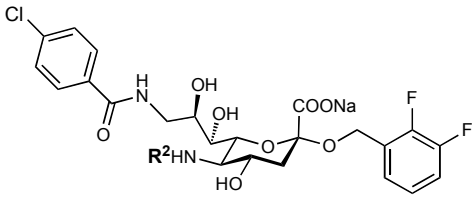
13a	
13b	
13c	
13d	
13e	
13f	



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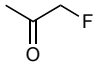
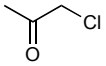
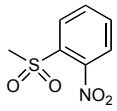
19a	
19b	
19c	
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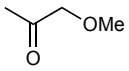
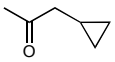
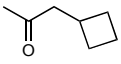
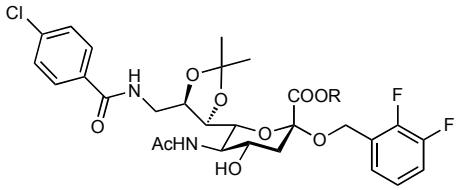
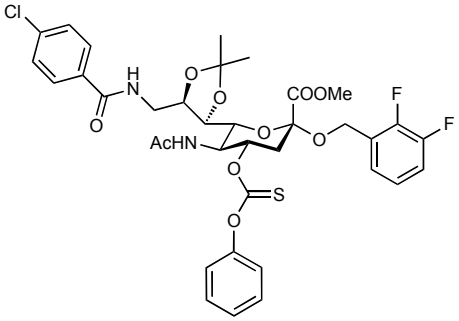
19e	
19f	
19g	

-Chapter 2.1.3-



10 R = CH_3
11 R = Na



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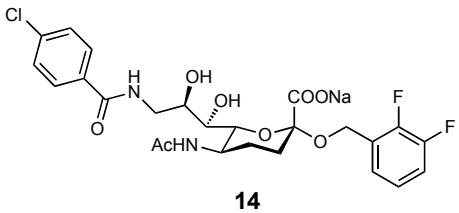

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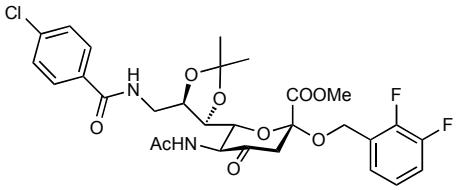

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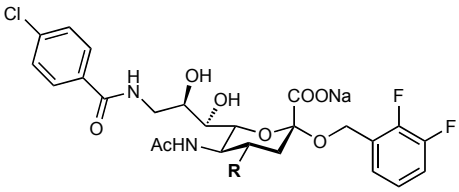

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	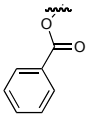	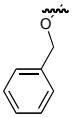	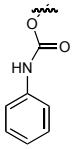	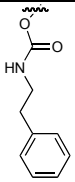	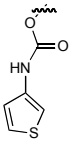	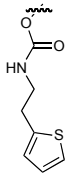	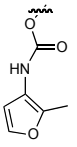

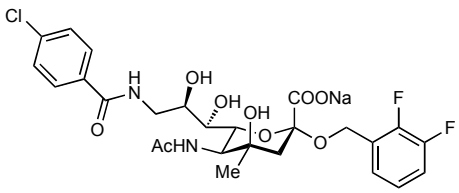

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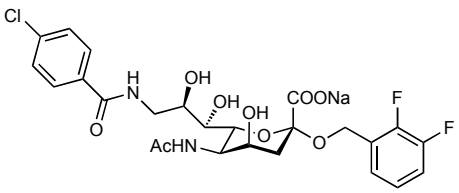

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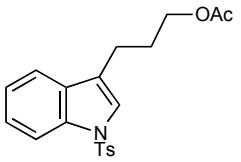
-Chapter 2.1.4-



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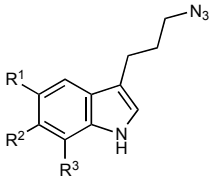
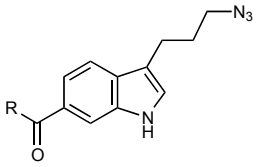


25a - k

a: R^1, R^2, R^3 = H
b: R^1 = OMe, R^2, R^3 = H
c: R^1 = Cl, R^2, R^3 = H
d: R^1 = F, R^2, R^3 = H
e: R^1, R^2 = H, R^3 = Me
f: R^1 = H, R^2 = Cl, R^3 = H
g: R^1 = isopropyl, R^2, R^3 = H
h: R^1, R^2 = -$(CH_2)_3$-, R^3 = H
i: R^1, R^2 = H, R^3 = Cl
j: R^1 = CN, R^2, R^3 = H
k: R^1 = SO_2Me, R^2, R^3 = H



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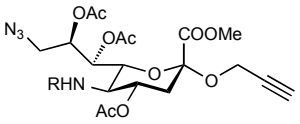
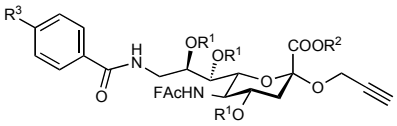
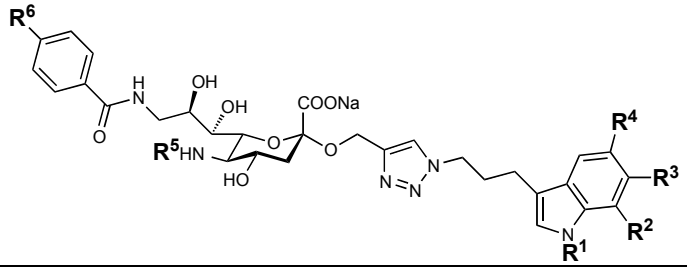
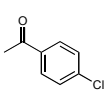


34: R = Boc
35: R = H
36: R = FAc



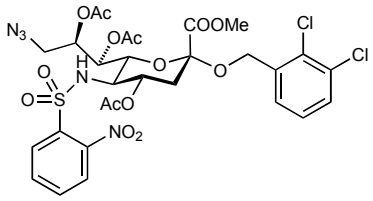
38a: R^1 = Ac, R^2 = Me, R^3 = H
38b: R^1 = Ac, R^2 = Me, R^3 = Cl
39a: R^1 = H, R^2 = Li, R^3 =H
39b: R^1 = H, R^2 = Li, R^3 = Cl



Compound	R^1	R^2	R^3	R^4	R^5	R^6
1	H	H	H	NO_2	Ac	H
2	Me	H	H	NO_2	Ac	H
3	Et	H	H	NO_2	Ac	H
4	H	H	H	H	Ac	H
5	H	H	H	OMe	Ac	H
9	H	H	H	CF_3	Ac	H
7	H	H		H	Ac	H
8	H	H		H	Ac	H

9	H	H	H	NO_2	FAc	H
10	H	H	H	NO_2	FAc	Cl
11	H	H	H	Cl	FAc	Cl
12	H	H	Cl	H	FAc	Cl
13	H	Cl	H	H	FAc	Cl
14	H	CH_3	H	H	FAc	Cl
15	H	H	H	F	FAc	Cl
16	H	H	H	OCH_3	FAc	Cl
17	H	H	H	$CH(CH_3)_2$	FAc	Cl
18	H	H	H	CN	FAc	Cl
19	H	H	H	SO_2Me	FAc	Cl
20	H	H	cyclopentane		FAc	Cl

-Chapter 2.2-



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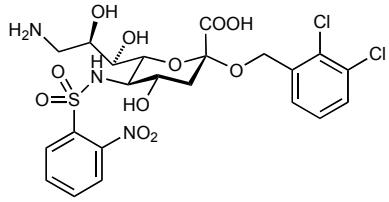

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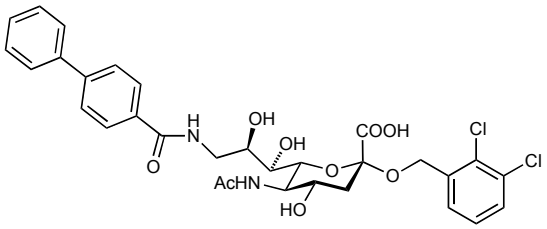
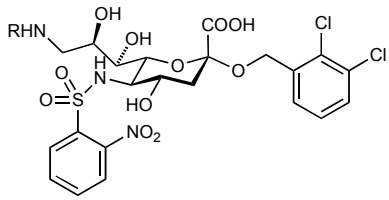
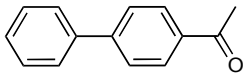
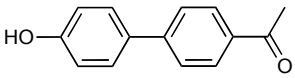
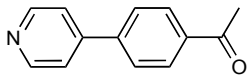
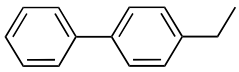
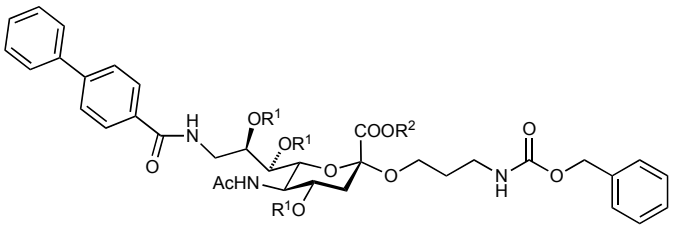


10b

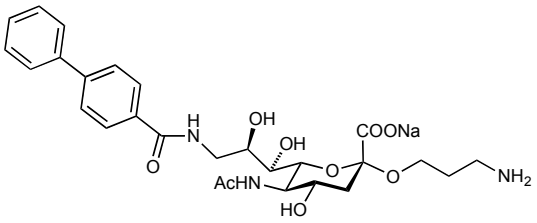


Compound	R
14a	
14b	
14c	
17	

-Chapter 2.4-



3: R^1 = Ac, R^2 = Me
4: R^1 = H, R^2 = Na



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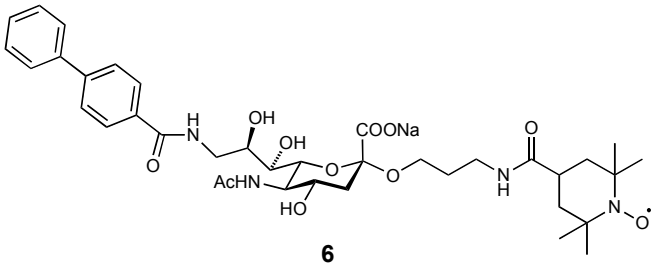
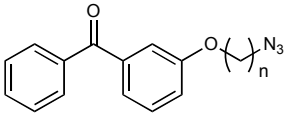
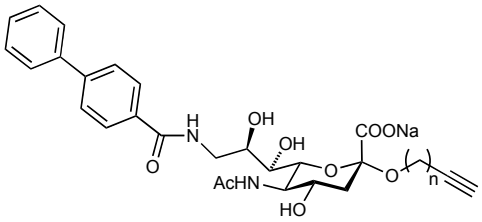


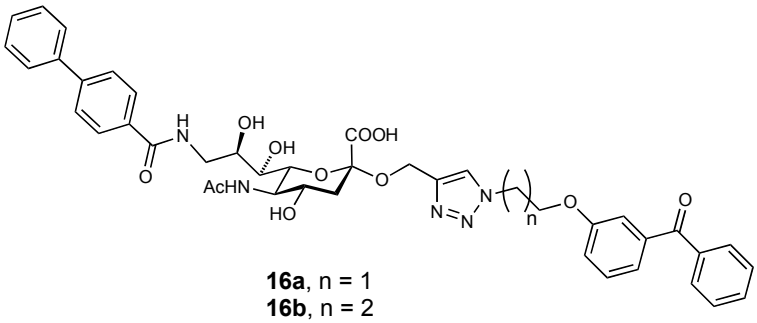
6



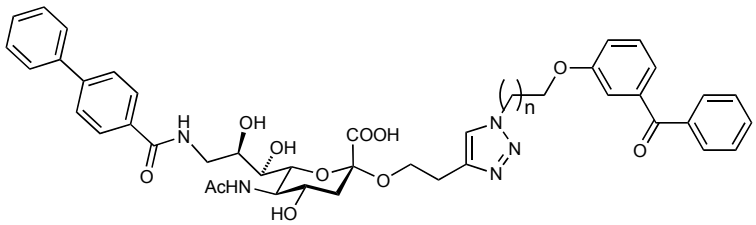
10: n = 2
13a: n = 3
13b: n = 4



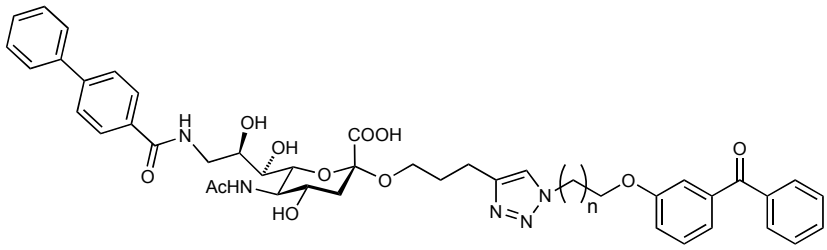
15a: n = 1
15b: n = 2
15c: n = 3



16a, n = 1
16b, n = 2
16c, n = 3



16d, n = 1
16e, n = 2
16f, n = 3



16g, n = 1
16h, n = 3

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Curriculum Vitae

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Education

01/2007-06/2010	PhD under the supervision of Professor Beat Ernst, University of Basel: **Synthesis and Biological Evaluation of Carbohydrate Mimetics as Ligands for Siglecs**
04/2006-11/2006	MSc in Chemistry under the supervision of Prof. B. Ernst, University of Basel: **Development of drug-like MAG-Antagonists**
2002-2006	BSc in Chemistry, University of Basel
2002	Abitur, Scheffelgymnasium Bad Säckingen, Germany

Professional skills

Synthesis	Synthesis of complex carbohydrate mimetics, transition metal catalysed reactions, stereoselective synthesis
Biacore	Assay development, performance and evaluation of measured data
Analytics	NMR, IR, HPLC

Other skills

Languages	German: mother tongue English: fluent French: intermediate knowledge
Computer	Knowledge of ChemDraw, MestreNova, Topspin Basic knowledge of Molecular Modelling (using MacBio/ MacYeti®) Knowledge of Microsoft Office
Scientific writing	Experienced in writing publications and scientific reports

Courses and workshops

2008	Swiss Course on Medicinal Chemistry, Leysin, Switzerland
	Lecture Notes on Structure-Based Drug Discovery, Part I/II, Dr. K. Müller, Roche, Basel, Switzerland
2007	Kinetic and Affinity Analysis using Biacore, GE Healthcare, Uppsala, Sweden
	Efficient writing course, Advanced Study Centre, Basel, Switzerland

Teaching

2007-2010	Management of weekly meetings of the Siglec project
2007-2009	Supervision of the course "Modern Drug Design" in Pharmaceutical Chemistry
2007	Supervisor of a Master thesis with the title "*Synthesis and Biological evaluation of Drug-like MAG-Antagonists*"

Interests

Sports	Running, climbing
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Publications

- **S. Mesch**, K. Lemme, M. Wittwer, H. Koliwer-Brandl, O. Schwardt, S. Kelm, B. Ernst, From a library of MAG-Antagonists to nanomolar CD22 ligands. *ChemMedChem*, **2011**, *accepted*.
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Presentations

- S. Mesch, **Kinetic and Thermodynamic Properties of MAG-Antagonists**, Eurocarb 2009, Vienna, Austria.
- S. Mesch, **Synthesis and Biological Evaluation MAG-Antagonists**, Annual Research Meeting, Basel, Switzerland
- S. Mesch, **Thermodynamic and Kinetic Considerations of the Binding Process of MAG-Antagonists**, SCS Fall Meeting 2007, Lausanne, Switzerland.

Posters

- S. Mesch, G. Rossato, K. Lemme, H. Koliwer-Brandl, A. Vedani, O. Schwardt, S. Kelm, B. Ernst, Poster No 149, ACS Anaheim, CA, 2011.
- S. Mesch, O. Schwardt, B. Ernst, Poster No. 587, ISCM Vienna, 2008.
- S. Mesch, M. Spreafico, A. Vedani, O. Schwardt, B. Ernst, Poster No 104, SCS Zurich, 2008.
- S. Mesch, D. Strasser, O. Schwardt, B. Ernst, Poster No. 90, SCS Zurich, 2006.

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